Characterization and mutational analysis of haemagglutinin and neuraminidase of H3N2 and H1N1pdm09 human influenza A viruses in Egypt

May S. Soliman 1 · Mahmoud M. Kamel 2 · Jamal A. Alorabi 3 · Nader M. Mohamed 4,5 · Amani A. El-Kholy 1 · Ahmed S. Abdel-Moneim 6,7

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Abstract Seasonal influenza viruses constitute a major global concern. Currently, H3N2 and H1N1pdm09 are the commonly circulating influenza A viruses. The haemagglutinin and neuraminidase genes of influenza A(H3N2) and A(H1N1)pdm09 viruses from Egyptian paediatric patients with respiratory distress were sequenced. Mutational analysis of all published sequences from Egypt was evolutionary tracked for both HA and NA genes. Phylogenetic analysis of H3N2 HA showed that the Egyptian strains belong to 3C2 subclade while Egyptian A(H1N1)pdm09 strains belong to 6B1 subclade. Some Egyptian A(H1N1)pdm09, 2013–2014, strains form a new subclade; 6B3. High score of mutations were recorded in HA of H1N1pdm09 but higher was recorded in H3N2 strains. These findings confirmed a high mutation rate of influenza A subtypes specially H3N2 strains.

Keywords H3N2 · H1N1 · Influenza · Mutational analysis · H1N1pdm09 · Egypt

Introduction

Seasonal influenza viruses continue to constitute a major global concern that is responsible for high morbidity (5–10% of the adults and 20–30% of children) and considerable mortality of approximately 290,000–650,000 deaths annually [27, 28]. Both influenza A virus (IAV) and influenza B virus (IBV) are the potential causes of seasonal influenza. Human influenza A viruses (IAVs) belong to the genus Alphainfluenzavirus within the family Orthomyxoviridae. Viruses of that genus possess eight segments of negative-sense RNA, PB2, PB1, P, HA, NP, NA, M and NS, which encode for ten essential proteins [1]. Both HA and NA proteins determine the antigenic properties and pathogenicity of the influenza viruses. HA is responsible for virus attachment, envelope fusion and neutralization while the virus uses NA for eluting the virus progeny from infected cells [1]. The HA protein is cleaved by cellular proteases into the HA1 and HA2. To date, there are eighteen different types of haemagglutinin (HA) and eleven types of the neuraminidase (NA) [1]. Although there are dozens of different subtypes of the IAV, only three IAV subtypes, H1N1, H2N2, and H3N2, have been reported to infect humans [31].

Currently, the circulating human influenza A viruses include H3N2 and H1N1pdm09. The latter replaced the seasonal influenza H1N1 virus which had circulated prior to 2009 [9, 27]. The current annual seasonal influenza vaccine is efficient in reducing the severity of the disease symptoms. It also guards against the considerable mortality especially in young children, elderly persons, pregnant

Ahmed S. Abdel-Moneim
asa@bsu.edu.eg; asa@tu.edu.sa

1 Clinical Pathology Department, Faculty of Medicine, Cairo University, Giza, Egypt
2 Clinical Pathology Department, National Cancer Institute, Cairo University, Giza, Egypt
3 Biology Department, College of Science, Taif University, Al-Taif 21944, Saudi Arabia
4 Department of Paediatrics and Neonatology, Al Ahrar Hospital, Zagazig, Egypt
5 Department of Paediatrics and Neonatology, College of Medicine, Taif University, Al-Taif 21944, Saudi Arabia
6 Microbiology Department, College of Medicine, Taif University, Al-Taif 21944, Saudi Arabia
7 Virology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni Suef 62511, Egypt
women and immunocompromized patients [15]. Accumulation of point mutations in the haemagglutinin (HA) leads to genetic drift and virus evolution that facilitates the escape from the host immune response with subsequent decrease in vaccine efficiency [22]. Neuraminidase (NA) is the second main surface protein that is also subjected to mutations that are recently linked to the mutation of HA [5]. Accordingly, continuous analysis of the sequence variations of the circulating seasonal influenza viruses is important for detecting variant strains. There is a shortage in the screening of influenza in Egypt a fact that diminish the understanding of influenza evolution and epidemiology [4, 16]. The current study aimed to molecularly characterize the influenza A viruses present in clinical samples from paediatric patients. It also intended to track the mutational changes of HA and NA genes in such strains and other Egyptian strains present in the EpiFlu database.

Materials and methods

Ethical approval

The study protocol was ethically approved by the Faculty of Medicine, Cairo University Ethical Committee. Written informed consents were collected from the guardian of the children.

Specimen collection

Sixty nasopharyngeal swabs were obtained from diseased children under the age of five years at the Paediatric Hospital, Aboelreesh, Giza, Egypt. The children suffered from influenza-like symptoms including fever, cough, sore throat and runny nose from Jan 2015 to Dec 2016. Each swab was kept in a universal virus transport medium (1 mL) and routinely processed. Processed samples were transferred to sterile RNase free tubes and stored at 

-80°C.

Nucleic acid extraction and real time RT-PCR

Viral RNA was extracted with 250 μL of the clarified supernatant of the nasopharyngeal fluid using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Viral RNA was used as a template for cDNA synthesis according to the manufacturer’s instructions (cDNA Synthesis Premix (Seegene, Korea). Samples were screened for influenza A virus, influenza B virus, adenovirus, parainfluenza viruses (1–4), rhinovirus, human respiratory syncytial virus (A and B), human bocavirus (HBoV), metapneumovirus, human coronaviruses (OC43, NL63, and 229E) and enterovirus (HEV) using respiratory virus detection kits-A and B (AnyplexTM II RV16 detection; Seegene, South Korea) according to the manufacturer’s instructions.

RT-PCR for amplification of HA and NA genes

Viral RNA from samples showed strong positive for influenza A virus (IAV) (n: 10) was used as a template for the amplification of HA and NA from the H3N2 and H1N1pdm09 strains. One-step RT-PCR (Verso 1-Step RT-PCR kit ReddyMix) was used according to the manufacturer’s instructions and specific reference oligonucleotides primers [26]. PCR amplicons were purified using QIAquick gel extraction Kit (Qiagen). BigDye Terminator Cycle Sequencing was used to determine the nucleotide sequence of the purified PCR amplicon using the same primers.

Phylogenetic analysis

MEGA 5.2 freeware was used to process the raw sequences obtained in the current study prior to GenBank submission (accession numbers MG745919–MG745928). Maximum likelihood method and 1000 bootstrap replications and Tamura–Nei substitution model was used to construct the phylogenetic tree of different HA and NA genes. Representative influenza sequences representative to different virus subtypes were obtained from the influenza databases (GISAID Epiflu and GenBank) and used in building the phylogenetic tree.

Tracking of mutations of HA and NA proteins

Comparison of amino acid sequences of HA and NA proteins of both Egyptian H1N1pdm09 and H3N2 available in GISAID Epiflu database in addition to the sequence obtained in the current study were conducted. All Egyptian H3N2 strains from 2006 to 2017 and H1N1pdm09 from 2009 to 2017 were included in the analysis. Deduced amino acids for different genes were created using MEGA 5.2. The amino acid substitution was also analysed in the Influenza Research Database (https://www.fludb.org/brc/home.spg?decorator=influenza) and the scores of variations were recorded. Scores ranged from 0 (no polymorphism) to 200 (highest polymorphism) as previously described [7].
Results and discussion

Real time qPCR on clinical samples and RT-PCR for IAV

In the current study, 26 out of 60 (43.3%) samples of clinical paediatric patients’ samples were found positive for influenza A: H1N1pdm09 (six samples; 10%) and H3N2 seasonal influenza A subtype (twenty samples; 33.3%). Mixed infections were recorded with H3 subtypes including human respiratory syncytial virus A (hRSVA) in four samples (6.7%). Other mixed infections with either bocavirus, human coronavirus 229E or adenovirus were also recorded in four cases (6.7%). HA and NA gene sequencing were successful from five out of ten samples that showed strong positive real time PCR: three H3N2 and two H1N1pdm09 strains.

Genotyping and mutational analysis of H1N1pdm09 strains

The genotype of the detected influenza A H1N1pdm09 strains, A/Egypt/BSU-13/2016 and A/Egypt/BSU-15/2016, detected from clinical cases of severe respiratory distress in Egypt, were found related to 6B1 subtype (Fig. 1a). Phylogenetic analysis of the neuraminidase of the same strains (Fig. 1b) revealed similar clustering profile to that of the HA (Fig. 1a). There are at least nine major genetic groups of H1N1pdm09 [14]. Since 2014, the genetic group 6 has prevailed. All the 2014–2016 Egyptian H1N1pdm09 strains found in the GISAID Epiflu database are related to genotype 6B and 6A but none related to 6C. Genetic group 6 harbours the amino acid residues characteristic to such genotype including: D97N, S185T, S203T, E374K and S451N. This group is subdivided into diverged into subgroups 6A (H138R, V249L), 6B (K163Q, A256T, K283E, E499K) and 6C (V2341, K283E, E499K). Interestingly, strains belong to 6C subgroup was not recorded in Egypt. Genotype 6B is further subdivided into 6B1(S84N, S162N) and 6B2(T13A, in the signal peptide and, N162S, N84S). In the current study, a newly detected 6B3 cluster was detected that contain signal peptide (L4T, T13A), N162S, N187S). Screening the score of variability of different amino acid residues of the current strains and those published in the influenza database, only 13 amino acid residues showed high score of variability, one in the signal peptide, and one in the S185T in Cb site (Table 1).

The HA1 subunit of the HA possesses the receptor binding site which is surrounded by five antigenic sites named: Sa, Sb, Ca (Ca1 and Ca2) andCb [30]. Antigenic drift at such amino acids leads to evolution of immune escape mutants or antigenic variants [23, 24]. These antigenic sites were found conserved in H1N1pdm09 Egyptian strains; however, considerable scores of mutations were detected in Ca site (D222E/G/N) Sb site (N162S, Q163K) and in Cb site (T185S) (Fig. 2). Ser 162 to Asn in the Sb site results in increasing the number of N-glycosylation site which is assumed to increase the virus virulence [21]. Increasing the affinity to α-2,6-linked sialic acids is expected by increasing the mutations in the H1N1pdm09 haemagglutinin [8]. Egyptian strains possess Asp 187 in all samples.
published isolates and Asp 222 in the majority of isolates (Fig. 2), such amino acid residue provide the affinity to the upper respiratory tract receptor, α-2,6-sialic acid [3].

Genotyping and mutational analysis of H3N2 strains

Both phylogenetic trees of the HA and NA of the H3N2 showed similar pattern of strains distributions (Fig. 1c, d). The characterized H3N2 in the current study and the Egyptian strains found in the different influenza databases during 2016–2017 were found to be related to subgroup 3C2. Such strains contain S45N and T48I (3C) as well as Q33R, N145S, N278K, D489N(3C2) (Table 2). They possess L3I, N144S, F159Y, K160T, N225D, Q311H (Table 2) which were found to be linked to 3C.2b subclade as previously described [14]. Two unique amino acid substitutions were detected in A/Egypt/BSU-8/2015 (H3N2): Y178D and N230T (data not shown) with yet unknown influence of pathogenicity or antigenicity.

The cell-based influenza seasonal vaccine (2016–2017) used a seed virus that had undergone egg passage that possessed T160K HA amino acid mutation assumed to be related to the egg passage [32]. This speculation could be not true since the T160K was found in most of the circulating Egyptian H3N2 strains. The majority of 2006–2014 strains (62/64) harbour T160 (Table 2). While 2015–2017 H3N2 strains, possess T160 (Table 2).

Five antigenic sites (A–E) are present in the H3N2 haemagglutinin: A (122, 124, 126, 131, 133, 135, 137, 142–146), B (155–160, 163, 186, 188–190, 192, 193, 196, 197), C (50, 53, 54, 275, 276, 278, 299), D (121, 172, 174, 201, 207, 213, 217, 222, 225–227, 242, 244, 248), E (57, 62, 63, 75, 78, 82, 83, 94) [12, 19, 20]. High score of mutations were recorded in the amino acid residues of epitopes: A (T135K, R142G/K/A, N144K/S, N145S), B (L157S, F159Y, K160T), C (D53N/G, N278K) and D (N121D/K/S, N225D) while low score of mutations were recorded with amino acid residues of epitope E (Table 2). It was presumed that the co-mutation of NA and HA of the H3N2 strains is utilized for the selection of the virus [5]. Amino acid combinational co-mutation in both HA and NA genes was detected mainly in H3N2 of the season 2013 and NA93G–HA278K was found to be predominant in Japan that was then in the following seasons, NA93D–HA278N was then become more evident. The latter combination was presumed to be more stable in structure than NA93G–HA278K [5]. It is known that amino acid residue HA 278 is associated with the epitope C [29] whereas; NA 93 is not related to any functional sites of the NA including the catalytic, antigenic epitope, or even the N-linked glycosylation sites [2, 6]. N278K is present in the H3 of 2015–2014 Egyptian isolates while the N278 was found in the H3 of 2006–2013 strains (data not shown). Amino acid mutation in the residue 43 of the NA was found to change the virus antigenicity and hence contribute to antigenic drift of the virus [17, 25].
Screening of possible drug resistant mutations in NA proteins

The clinical resistance to the neuraminidase inhibitors were found to be associated with Arg 292 to Lys and Glu 119 to Val amino acid substitution in H3N2 while His 275 to Tyr and Asp 294 to Ser amino acid substitutions in H1N1 virus subtypes [10, 13]. All the Egyptian H3N2 strains harbour Arg at 292, and Glu at 119 while Egyptian H1N1pdm09 possess His 275 and Asp 294 except for a single strain found in the database (EPI824334/A/Egypt/1424/2016) that possesses 275 Tyr). Data not shown). Meanwhile,
neuraminidase D79G, and S247N amino acid substitutions of the H1N1 neuraminidase were associated with oseltamivir resistance [18] while H126N or Q136K were found to confer resistance to zanamivir [11, 18]. None of these mutations were found in the Egyptian H1N1pdm09 strains found in the database.

It was concluded that detected H3N2 and A(H1N1)pdm09 Egyptian strains belong to 3C2 and 6B1 subclades, respectively. Some A(H1N1)pdm09, from 2013 to 2014, constitutes a new subclade; 6B3. High scores of amino acid substitutions were detected in both HA of H1N1pdm09 and H3N2 however, higher scores were found among H3N2 strains.

Compliance with ethical standards

Conflict of interest

The authors declare that there is no conflict of interest.

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