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Identification of Non-Haemagglutinating Influenza A/H3 Virus and Characterization of Haemagglutinin (HA) and Neuraminidase (NA) Strain Mutations in Influenza-Like-Illness Cases in Egypt on MDCK Cell Line.

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ABSTRACT

Haemagglutination Inhibition assay "HAI" is one of the routinely used assays in the Influenza virus isolation workflow. Many Influenza A/H3 isolates can be mistakenly considered negatives if HAI is the only tool for culture identification, due to the increase in numbers of non-haemagglutinating influenza strains circulation.

We wanted to explore the presence of non-haemagglutinating influenza A/H3 in Egypt and to discover potential mutations.

481 Oropharyngeal swabs in 2014 were collected from outpatients with Influenza-like-illness from eight hospitals in Egypt.

Samples were tested for influenza viruses by qRT-PCR, then virus isolation. Cultures were tested by HAI, indirect Immunofluorescence assay "IFA" and Sequencing of HA and NA genes.

QRT-PCR identified 84 Influenza A/H3 samples, 12 of which were successfully cultured and confirmed by qRT-PCR, IFA, and sequencing.

Results of HAI showed Six Haemagglutinating and Six non-haemagglutinating cultures. HA and NA sequencing revealed mutations present in non-haemagglutinating and absent in Haemagglutinating isolates in both HA and NA genes.

We could link only one mutation per gene HA (N225D) and NA (D151N) to the agglutination avidity of Influenza A/H3 isolates. We suggest that the loss of ability is due to the N225D mutation in non-haemagglutinating isolates causing steric hindrance nearby the binding site of HA spikes. Whereas the agglutination capacity of haemagglutinating isolates is increased due to D151N found in the haemagglutinating only, suggesting the agglutination through the NA spikes also as referenced in literature.

As we have confirmed the presence of non-haemagglutinating influenza A/H3 in Egypt we recommend the use of IFA as the confirmatory assay in the Influenza virus isolation workflow, recommendations also for further to study virulence and prevalence of the identified virus.

INTRODUCTION

Influenza virus is one of the widely known respiratory pathogens that have been always a pathogen of interest to the public health field and a global topic of study for its emerging properties and its previous responsibility for several worldwide outbreaks in addition to the high morbidity correlated to the virus each year.

Each new characteristic or loss of a known viral characteristic comprises a very important topic of a study and requires a detailed approach to track any changes in both the genetic perspective and the epigenetic characteristics (Sharma V. *et al*., 2016).

The HA spikes are responsible for the virus attachment to its host cell, and then it mediates the release of the viral nucleoproteins into the cytoplasm of the host cell. Species specificity of each virus depends on the proper spike-receptor matching, as HA targets a specific sialic acid receptor on the target host cell, this selective process determines the susceptible species and organs which can be infected by the virus (Das *et al*., 2010).

Virus isolation and propagation is a crucial step for the global Influenza network tracking of circulating strains in the northern and southern hemispheres. Since mid 70's, 1975, the use of Madin-Darby Canine Kidney (MDCK) cells has been widely used in culturing the Influenza viruses for In-vitro propagation (Tobita K. *et al*. 1975) (Matrosovich *et al*., 2003) (Asghar A. *et al*., 2016).

Since 1992, the predominant circulating Influenza A strain became (H3N2) specially in Europe and North America, and most of these circulating H3N2 Subtype are found to lose the ability to agglutinate Chicken, and Turkey erythrocytes recommended to carry out the HA test *(Gulati et al., 2013; Ito et al., 1997; Lin et al., 2012, Nobusawa et al,. 2000; Medeiros et al. 2001, Fleming DM et al. 2008, WHO 2012)*, studies for a definitive explanation on the exact reason and genetic mutation responsible for such modification is still under investigation although different suggestions had been placed and correlated to this phenomena, yet, there is no clear and exact factor is proven to be the only factor responsible for such behavior. point mutations were correlated and attributed to the loss of the ability to agglutinate avian red blood cells, mainly around the binding and fusion sites of both HA1 and HA2 proteins of the HA spike.

In this study, we aim to screen for the presence of non-agglutinating influenza A/H3 in Egypt and to explore the status of point mutations previously described in the literature that are correlated to changing agglutination ability towards avian erythrocytes.

MATERIALS AND METHODS

RNA Extraction and PCR Screening:

In this study, Oropharyngeal (OP) swab in viral transport media (VTM) were collected by the Egyptian Ministry of Health through the National Influenza Surveillance program started since 2009. Samples collected from outpatient of 8 sentinel sites in Egypt during 2014 were selected for this study. The8 sentinel sites are Gabarty Medical Center, Alexandria, Helwan, Imbaba, Zagazig, Menya, Damietta, and Aswan Fever hospitals. Patients with Influenza-Like Illness (ILI) case definition as identified by the WHO to be "fever ≥ 38°C and cough with onset within the last 10 days" were enrolled in this surveillance by collected an Oropharyngeal swab from each patient and place it immediately in 1ml Viral Transport Medium (VTM) in Cryovials. Samples were frozen and stored in liquid nitrogen tanks until delivered on weekly bases to NAMRU3 for testing andlong term storage in the repository of NAMRU-3 at -80°C in ultra-low freezers. A total of 481 OP swab samples were selected for these studies that were collected within a 3 months period during the Influenza season of 2014 in Egypt.

RNA extraction using QiaAmp mini RNA extraction kit (QiagenCat# 52906) was performed on all samples collected and were screened by Polymerase Chain reaction (PCR) using ABI7500 PCR machine (Applied Biosystem) according to the Center for Disease Control and Prevention "CDC" Influenza diagnostic protocol (Reference #K130551) for Influenza A and B. Influenza

A positive samples were further characterized by qRT-PCR for sub-typing, tested for seasonal A/H1 and H3, PdmH1 and Avian influenza H5 in parallel.

Cells and Virus Isolation:

Positive PCR samples for influenza A/H3 were inoculated into MDCK (Madin-Darby Canine Kidney cells, ATCC # CCL-34) cell line susceptible to Influenza virus propagation. Cells were checked daily and inspected for Cytopathogenic effect (CPE), positive CPE samples were tested by qRT-PCR for the presence of Influenza A/H3 and screened with HA assay, the culture supernatant was also re-inoculated into new MDCK cells for confirmation. Cultures of the second passage were also checked daily for the presence of CPE as an indication of viral growth. Confirmed CPE for 2 successive passages was screened by Hemagglutination assay (HA) and Hemagglutination Assay Inhibition (HAI) using the WHO Influenza reagent kit for the identification of influenza isolates (WHO Global Influenza Surveillance Network, 2011). Viral isolates showing negative HA results were re-inoculated into new MDCK tubes for CPE confirmation, HA was repeated to assure negative results.

All CPE positive samples in the second passage (Both batches, those with positive HA results and negative HA ones) were tested by IFA (Indirect Immunofluorescence assay) against Influenza A andB types and also for Influenza A subtyping against both Influenza A/H3 and Influenza A/PdmH1 monoclonal specific antiserum.

qRT-PCR was also performed to confirm the presence of Influenza A/H3 viral RNA in culture, All Influenza A/H3 viral cultures including HA and non-HA cultures from the first passage were amplified and HA and NA genes were sequenced using Sanger sequencing following CDC influenza A/H3 sequencing protocol.

RESULTS

481 Oropharyngeal (OP) swab samples, collected from patients with ILI case definition, in 8 sentinel sites in Egypt during influenza season of 2014 “October - December "were selected for the present study.

QRT-PCR identified 93 Influenza A and 17 Influenza B infections with one case of co-infection of both Influenza viruses A and B. From the 93 Influenza A samples, 84 were subtyped as influenza A/H3 while 9 were found to bePdmH. The sample with the co-infection was positive for influenzaA/H3 subtype with a low CT value (CT= 38). Virus culture of InfluenzaA/H3 samples showed positive Cytopathogenic effect (CPE) in 12 samples of the 84 inoculated samples for 2 successive passages, while 3 samples only showed positive CPE from the 9 influenza A (PdmH1) samples. From the 12 influenza A/H3 positive cultures that were confirmed by qRT-PCR, only 6 samples showed positive HA results with TRBC’s, while the other 6 showed negative agglutination ability.

All culture cell supernatant samples processed for indirect Immunofluorescent assay (IFA) showed positive fluorescence results against Influenza A type and Influenza A/H3 subtype monoclonal antibodies (Cat# 3105, respiratory panel, light diagnostics), and showed negative results for adenovirus, influenza B, parainfluenza 1, parainfluenza 2, parainfluenza 3, and respiratory syncytial virus (RSV), while all influenza A/H3 samples showed positive IF results to Influenza A antibody. We used specific Antibody for influenza A/H3 virus (Cat# MAB8254, Chemicon) using the same IF procedures, the 12 influenza A/H3 cultures showed positive IF reaction. Positive cultures were confirmed by HA sequencing to be Influenza A/H3, HA gene analysis sequence of 4 samples (out of the 6 Non-Haemagglutinating influenza A/H3 isolates) were successfully performed, while 3 only were successfully sequenced for the NA gene. Non Heamagglutinating samples were tagged as NHA followed with the last 4 digits of their accession numbers.

HA and NA genes of a reference sample of Haemagglutinating isolate were

sequenced also for genetic comparison between non-Haemagglutinating against the Haemagglutinating isolates. The sample was referred to as Haemagglutinating (HA) followed by the last 4 digits of the accession number.

Results of HA Gene Sequences:

HA gene sequences for the 4 non agglutinating isolates (NHA-4842, NHA-4902, NHA-4940, NHA-5000) and the agglutinating isolate (HA-4870) were aligned and checked for consistent changes, certain positions of interests were compared according to previous references found in literature, result of the selected amino acid position, that was suspected to show mutational changes between agglutinating and non-agglutinating, results at the HA protein in amino acids 81, 98, 112, 133, 145, 173, 190, 193, 194, 222, 225, 226, 227, and 238 are shown in the below table:

Table 1: Comparison between agglutinating (HA) and non agglutinating (NHA) amino acid sequences of the HA protein at the expected point mutations (Table showing the positions we were expecting first to have mutations).

	Positions on HA gene													
	81	98	112	133	145	173	190	193	194	222	225	226	227	238
HA-4870	N	Y	V	N	S	Q	D	F	L	R	N	I	P	K
NHA-4842	N	Y	V	N	S	Q	D	F	L	R	D	I	P	K
NHA-4902	N	Y	V	N	S	Q	D	F	L	R	D	I	P	K
NHA-4940	N	Y	V	N	S	Q	D	F	L	R	D	I	P	K
NHA-5000	N	Y	V	N	S	Q	D	F	L	R	D	I	P	K

There was no difference found at the position 81, 98, 112, 133, 145, 173, 190, 193, 194, 222, 226, 227, and 238 of the HA protein between the non- agglutinating 4 isolates and the agglutinating isolate, but at the position 225 of the HA protein, a consistency of D was found at the 4 non agglutinating isolates where at the same position of the HA protein, and N was found at the agglutinating isolate.

When aligning the sequences of the 4 non-agglutinating isolates to the agglutinating isolate, a consistent difference has been detected in the 4 non-agglutinating isolates different than the agglutinating one in the HA protein in the following positions: 3, 62, 83, 122, 128, 142, 144, 157, 159, 225, 311, 347, and 489, as follows in the below table:

Table 2: Sequencing results of the HA gene showing differences between agglutinating (HA) and non agglutinating (NHA) amino acid sequences.(Table showing all difference found after alignment).

	Positions on HA gene												
	3	62	83	122	128	142	144	157	159	225	311	347	489
HA-4870	L	K	R	D	A	G	N	S	F	N	Q	K	D
NHA-4842	I	E	K	N	T	R	S	L	Y	D	H	V	N
NHA-4902	I	E	K	N	T	R	S	L	Y	D	H	V	N
NHA-4940	I	E	K	N	T	R	S	L	Y	D	H	V	N
NHA-5000	I	E	K	N	T	R	S	L	Y	D	H	V	N

Results of NA Sequences:

Results of NA sequences of 3 non-agglutinating isolates (NHA-4842, NHA-4902, NHA-4940) and the agglutinating isolate (HA-4870)were studied especially at the positions reported previously in the literature to carry mutations which are 93, 150, 151, 194, 310, 370, 372, and 387 in the NA protein. Of the 8 selected positions, the amino acid in position 151 of the NA protein showed the presence of D in the 3 non-agglutinating isolates while N at the same position of the agglutinating isolate, the result of the selected positions is as follows:

Table 3: Comparison between agglutinating (HA) and non agglutinating (NHA) amino acid sequences of the NA protein at the expected point mutations.

	Positions on NA gene							
	93	150	151	194	310	370	372	387
HA-4870	G	R	N	I	H	S	L	K
NHA-4842	G	R	D	I	H	S	L	K
NHA-4902	G	R	D	I	H	S	L	K
NHA-4940	G	R	D	I	H	S	L	K

After alignment of the 4 NA sequences, a consistent difference has been detected in the 3 non-agglutinating isolates and different than the agglutinating isolate in the NA amino acid sequence of the NA protein. The amino acid differences were found at positions: 55, 151, 155, 221, 251, 312, 315, 358, and 392. Whilethere were 2 positions where only 2 isolates of the non agglutinating showed the difference to that of the agglutinating isolate, and sequencing data of the 3rd non agglutinating was inconclusive at these 2 locations, 267, and 380, results are as follows in the below table:

Table 4: Sequencing results of the NA protein showing differences and deletion in amino acids in some positions of the NA protein of the agglutinating (HA) versus the non-agglutinating (NHA) amino acid sequences.

	Positions on NA gene																
	26	55	71	151	152	155	215	221	251	267	311	312	315	329	358	380	392
HA-4870	I	S	T	N	R	F	V	E	V	T	S	V	G	N	D	I	M
NHA-4842		P		D		Y		D	D	K		I	S	K	N	V	I
NHA-4902	T	P		D		Y		D	D	K		I	S		N	V	I
NHA-4940		P	I	D	K	Y	I	D	D			I	S		N		T

DISCUSSION

Evolution and continuous changes in the HA gene of the influenza viruses in particular is based on the fact of escaping antibody neutralization of selective mutants and ability of the mutants to cause an infection and amplifies, whereas the regular strains are neutralized and do not predominate as the circulating strain, so this explains the main variations mainly in the binding sites of the virus and is referred to as "Adsorptive mutants" as presented by Fazekas *et al*. (1977).

According to several studies on the agglutination ability of the H3 viruses to agglutinate RBC's and attachment affinity to receptors, many factors were suggested to be responsible for the influenza A/H3 virus evolution. Most mutations correlated are present in the HA protein mainly around the 190 or 220 regions, some other mutations were found around the 90 and 150 regions of the NA protein.

Mutations were either correlated to the change in binding affinity to a human receptors; so viruses would bind less to the target receptors found on specific RBC's having the same receptors, and other studies have directly linked the mutations of the HA spike leading to a change to the agglutination abilities towards certain species, as the chicken and Turkey RBC's.

Mutations Linked to Change in the Binding Affinity:

In 2010, Yi Lin proved that not only the HA gene was found to have a role in the binding affinities as expected, but also the substitution of amino acid 151 on the NA protein from Aspartic acid to Glycine, Asparagine, or Alanine and their effect on the HAI results of (151mutant H3 viruses) due to the unexpected role of viral attachment through the NA protein to the receptors instead of HA spikes, such mutation was found to have an impact on the vaccination outcome and represents difficulty in using HA/HAI assay as a diagnostic assay, since a positive HA agglutination result for a viral culture, would show a negative inhibition, as the binding of

the 151 mutant virl is through the NA spike, while the antiserum is mainly directed towards HA spikes.

Results showed that there is a consistency in the NA sequence at the 151 positions in the 3 non-agglutinating isolates different than the agglutinating isolate. Aspartic acid was found in the 3 isolates while Asparagine is found instead in the agglutinating isolate, as previously discussed, the presence of Asparagine at the 151 positions of NA protein of the agglutinating isolate only suggests the possibility of virus- receptor attachment through the NA spike as suggested by Yi Lin (2010).

Mutations Linked to the Loss of Agglutination Ability:

The mutation in the HA gene leading to the alteration of the amino acid sequences "Glu190Asp" has been suggested as one of the important mutations linked to the loss of the agglutination ability (Nobusawa *et al*, 2000), in addition to the successive mutations at position 226 from Gln226-to-Leu-to-Ile-to-Val (Medeiros *et al*, 2001). Both mutations are found to be present at positions 190 and 226 in both agglutinating and non-agglutinating isolates, where Asp is found at position 190 in the 5 isolates (both agglutinating and non-agglutinating), and Ile is found in the 5 isolates, suggesting the loss of ability to agglutinate RBCs as documented in the references through the HA spike of theinfluenza A/H3 influenza virus.

The main domain that was correlated before to the loss of the agglutination properties is the 220 Loop of the HA1 protein, around the binding site of the HA, most of the mutations related were around this region. Several single and co-mutations appeared. According to Lin. *et al*. (2012), changes in positions 225 and 226 are thought to be related to the loss of the avidity towards avian blood agglutination. In addition to Trp-222-Arg, previous changes published before was found to be Gly-225-Asp for samples collected between 2001-2002, with a Leu226Val in the HA gene, where in samples from 2004, changes became Asp-225-Asn, and Ser193Phe (with Arg still present in 222) and a Val226 ile.

Our results showed the presence of the Arg at position 222 in the 5 isolates, Phe at position 193 also in the 5 isolates, while at position 225, there was no Gly available but Asp is present instead at the 4 non-agglutinating samples, suggesting a similarity to those samples collected between 2001-2002, while the agglutinating sample showed Asn at position 225, suggesting the similarity to the samples collected after 2004, these suggestions supports the idea of having different strains of the virus rather than one strain with minor differences.

Conclusion and Recommendations:

In conclusion, through the efforts of influenza surveillance in Egypt to monitor the disease burden and identify circulating strains in the country, we have identified the presence of non-heamagglutinating Influenza A/H3 viruses in Egypt and screened for the presence of several point mutations according to previous literature correlated with the modification of binding avidity to the RBC's. We have detected several suspected point mutations other than the ones documented in literatures before, which requires a specific deeper study before linking them with the modification of agglutination abilities. The main position, which was previously documented to be responsible with the loss of ability and we suggest that it's the responsible for non-agglutination strain in Egypt is the N225D on the HA protein which is a consistent change in the non-agglutinating isolates in comparison to the agglutinating isolates.

As per the identified Asp 151 Asnmutation in the NA amino acid sequence of the agglutinating isolate, as discussed previously this mutation that has been referred to increase the attachment ability of the NA spike to the SA α 2, 3 Gal receptor on host cells and Turkey red blood cells, such attachment leads to successful Turkey red blood cells agglutination, but an attachment through NA spike is not the regular route of viral infectivity, an

attachment of which may be responsible of an unsuccessful infectivity or at least lowering down the number of infective viral particles during the infection, we recommend to study more the effect of this modification on the infectivity cycle of the virus along with the virus from previous year. .However; the effect of neuraminidase on agglutination was not studied during the progress of this research, which is a limitation to our conclusion

IFA or any other confirmatory assay that does not depends on agglutination ability but rather depends on virus antibody interaction such as ELISA ormicro-neutralization assay is recommended to be used as a routine confirmatory assay in the Influenza virus isolation workflow in general and in Egypt in specific, considering the presence of circulating non-Haemagglutinating Influenza A/H3 in the country, HA/HAI can't be the sole screening and identification tool in virus isolation procedure.

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