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Efficacy of Composting Poultry Mortality and Farms Wastes with Mixed Respiratory Infection Viruses H9N2 and NDV in Egypt

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Abstract: Composting poultry mortality and farm wastes infected with mixed respiratory infection viruses one of major goals for control and combating this infection. To achieve fair level of biosecurity protocol in poultry farms infected with virus induced mortality, proper hygienic disposing is imperative from socioeconomic and health risk concept. Collected fresh dead birds, their litter and wastes from commercial flocks with high mortalities associated with mixed respiratory symptoms were subjected to composting process. Molecular characterization of avian influenza virus (H9N2) and Newcastle disease virus (H9N2 & NDV) were recorded from cecal tonsils and trachea respectively of morbid and dead chickens before subjecting to composting. Characterization was done by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using specific primers targeting the matrix (M) gene, H9 gene of AIVs and fusion (F) gene of NDV were used. The (HA) hemagglutinin glycoprotein gene of H9N2 isolates were partially amplified by RT-PCR, directly sequenced. The nucleotide and amino acid sequence analysis of the hemagglutinin gene of the characterized Egyptian viruses showed the highest similarity with one group of recent Israeli circulating strains. The Phylogenetic analysis for HA gene of H9 AIV showed the placement of the Egyptian viruses within the same lineage of H9N2 viruses that circulated in the region from 2006 especially with recent Israeli strains of G1 lineage (group B). Failure of re-characterization of AIV (H9N2) and NDV in the current work on day 15th of composting treatment confirmed the efficacy of composting poultry farm mortalities and wastes. Composting in closed vessel (newly designed closed copmposter) achieved proper secure microbial activity that inactivated H9N2 AIV and NDV viruses via increased temperature and decreased moisture content of composting poultry mortality and farms wastes with no isolation and characterization of these viruses. The product suggested to be used for agronomic activities.

Key words: Composting Mixed Infection · NDV And H9N2 AIV

INTRODUCTION

H9N2 subtype virus is a notable member of influenza A genus as it can infect not only avian species but also, although sporadically, mammals such as pigs and humans [1]. Avian influenza viruses (AIV) and Newcastle disease virus (NDV) have been recognized as the most important pathogens of chicken inducing acute respiratory tract infections. Single-virus reverse transcription-polymerase chain reaction (sRT-PCR) assays are used extensively to detect these viruses in clinical samples [2]. Field outbreaks raised the concern for regarding the protective immunity of commercially available vaccines for prevention and control of H9N2 and NDV viruses in the field. An intensive vaccination program was implemented with live and inactivated vaccines which didn't guarantee control disease and virus combating without strict biosecurity measures employed [3, 4]. The advantageous growth of NDV may inhibit AIV propagation yielding false-negative AIV results when present in fecal samples [5]. In the case of AIV, it is not only of importance in causing illness and death to

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chickens, but also as a threat to human health. Viral interference is important to be interpreting by evaluating AIV and NDV replication using a new detection technique, real-time RT-PCR [6]. One of the major objectives of the eradication program of avian influenza virus is prompt and effective disposal of contaminated material that cannot be effectively treated like dead birds, litter, manure and fresh or frozen carcasses as know as composting. Composting has proven to be an environmentally sound method for disposing dead bird. The composting process management and monitoring thermal profile, moisture content, nutrient ingredients were recorded during day 1st to 33th and proved inactivation of H5N1 AIV from poultry mortality and farm wastes due to increased temperature 40-60°C during days 5-15th [7-9]. Composting has been used for disposal of poultry carcasses and manure following outbreaks caused by avian influenza virus (AIV) and Newcastle disease virus (NDV), but methods are needed to test the presence of these viruses in compost to ensure Biosecurity. Extracting viral nucleic acids from compost using RT-PCR method is an alternative for the virus isolation method for rapid detection and accurate quantification of AI and ND viruses in compost [10]. The first stage of the composting process must be performed in-house or in a reactor to avoid heat loss and further spread of the pathogen via vector animals, birds and rats. Thus, there is an increasing need for a safe sanitization method suitable for both small-scale and large-scale poultry production units. When comparing composting with other options such as incineration, composting can be regarded as a relatively economical and environmentally friendly alternative way [11]. To achieve fair level of Biosecurity protocol in poultry farms infected with virus induced mortality, proper hygienic disposing is imperative from socioeconomic and health risk concept. The current experiment was conducted to evaluate the efficacy of composting process as a method of hygienic disposal of poultry mortality and their wastes with mixed respiratory infection on the survival of AIV H9N2 and NDV in commercial poultry farms in Egypt during the last months of 2012.

MATERIALS AND METHODS

Source and Collection of Specimens: During the last months of 2012, commercial chicken flocks were suffered from high mortalities around Giza. Farms were visited and detailed history of the flocks including feed, medication, vaccination schedule and management conditions of the farms were noted. Those flocks were suffered from respiratory distress including rhinitis, gasping coughing, conjunctivitis and ocular discharge. The postmortem cross lesions were severe congestion of trachea with mucopurelant exudates, pericarditis and intestinal congestion. Samples were collected from freshly dead, allied and morbid birds for laboratory investigations. Samples were collected in sterile PBS, transported in cooler to the laboratory and kept in freezer at -70°C [12], until processed for molecular diagnosis [13].

Bulk Poultry Wastes from Poultry Houses: The type of collected poultry wastes was built-up litter including dropping, waste food, feathers, detached tissues and droppings. The dry straw bales were bought from private source to be used at base layer as well covering final layer, according to need for proper moisture content of composting mix. Random subsamples of poultry wastes from each poultry houses were collected and combined to yield a 0.5m^3 compost bulk sample of each poultry waste source [14]. The aseptically collected samples were sent to Virology laboratory (Faculty of Veterinary Medicine, Cairo University) for detection and confirmation of presence or absence of H9N2 and NDV viruses before and after composting.

Laboratory Investigations: Random samples 500-g of the compost mixes were removed from days 1 to 30th according to thermal phases (temperature increased up to 40-60°C) and sent to Virology Laboratory Faculty of Veterinary Medicine, Cairo University for isolation and characterization of viruses induced recorded respiratory disorders and mortality form examined poultry farms before and after composting process.

Daily monitoring of the temperature °C and relative humidity % were carried out as described by Zakia *et al*. [9]. Maintaining good management practices throughout the entire composting process helps ensure the elimination of insect larvae and pathogens in the final product [15].

Molecular Characterization of H5N1 and H9N2 AIVs

Viral RNA Extraction: Viral RNA was extracted from a pool of tracheal and cecal tonsils samples by using a Biozol RNA extraction reagent, according to the manufacturer's instructions (BioFlux, China). The method was followed up the guanidium-acid-phenol extraction method originally developed by Chomcznski and Sacchi [16]. All the procedures carried out as recommended by the kit instruction manual.

Table 1: Primer sets to amplify type-specific matrix (M) gene of AIVs, subtype-specific (H9) genes of AIVs and type specific Fusion (F) gene of NDVs

Name Sequence (50-30)	Expected product size
*MF TGA TCT TCT TGA AAA TTT GCA G	270bp
MR TGT TGA CAA AAT GAC CAT CG	
*H9F CTY CAC ACA GAR CAC AAT GG	487bp
H9R GTC ACA CTT GTT GTT GTR TC	
# NDVF TGG AGC CAA ACC CGC ACC TGC GG	766bp
NDVR GGA GGA TGT TGG CAG CAT T	

*Matrix (M) gene specific primers and H9 subtyping specific primers were designated by VRLCU (Virology Lab of Cairo University).
#Fusion (F) gene type specific primers of NDV were used as described by Mase *et al*. [20].

Primers Design and RT-PCR Reaction: RT-PCR reaction was performed separately for each virus using Promega Access RT-PCR System in a 50 ul reaction as described by Chaharaein *et al*. [17]. Specific primers targeting the matrix (M) gene, H9 gene of AIVs and fusion (F) gene of NDV were used (Table 1). The RT-PCR reaction tubes were incubated in the thermal cycler (Gene Amp® PCR system 9600; Applied Biosystems)) machine. The samples were partially amplified using the following conditions: RT at 48°C for 45min, one cycle at 94°C for 2 min, 40 cycles of heat denaturation at 94°C for 30s, primer annealing at 48°C for M gene, 50°C for both H9 & NDV for 1 min, primer extension at 68°C for 1min and one cycle of the final primer extension step at 68°C for 7 min. RT-PCR products (5ul) were visualized by electrophoresis in 1.5% agarose in 1X TAE, ethidium bromide was added to a final concentration of 0.5µg/ml and the gels were photographed. The remaining of PCR product volumes of H9 AIV were used for sequencing.

Sequencing and Phylogenetic Analysis: Gene sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) as described elsewhere. BioEdit software version 7.0.9.0was used to analyze and edit the generated sequence of the H9 gene of AIV. The GenBank database was screened (BLAST) for closely related sequences [18]. A Phylogenetic analysis of the newly obtained nucleotide sequence Using MEGA 4.0.2, the Phylogenetic tree was constructed by the neighbor-joining method and the reliability of each tree branch was estimated by performing 1000 bootstrap replicates [19]. The sequence of H9 AIV was submitted to GenBank under the following accession numbers: KC699553.1.

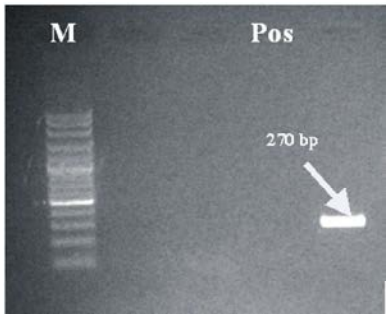

Fig. 1: Ethidium bromide agarose gel electrophoresis of the RT-PCR products of M gene of AIVs before composting. Lane M: marker 100bp with bands from lower to upper of the gel (100-200-300-400-500-600-700-800-900-1000-1200-1500-2000-3000) to the right M Lane Pos: represents the tested positive sample with expected correct size 273 bp

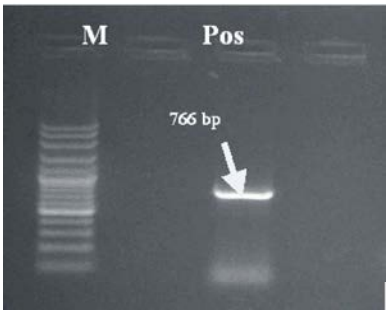

Fig. 2: Ethidium bromide agarose gel electrophoresis of the RT-PCR products of Fusion (F) gene of NDV before composting. Lane M: marker 100bp with bands from lower to upper of the gel (100-200-300-400-500-600-700-800-900-1000-1200-1500-2000-3000) to the right M Lane Pos: represents the tested positive sample with expected correct size 766 bp

RESULTS AND DISCUSSION

The molecular characterization of current H9N2 AIV was described from commercial chicken flock suffering from respiratory distress and was co infected with NDV confirmed with RT-PCR for Matrix (M) and HA genes of H9N1 AIV and fusion (F) gene of NDV (Fig. 1, 2 and 3) assay causing morbidity reached 70% and mortality reached 15%. Here we apply inactivation of H9N2 AIV

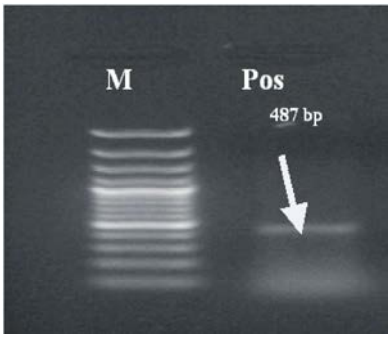

Fig. 3: Ethidium bromide agarose gel electrophoresis of the RT-PCR products of H9N2 AIV before composting. Lane M: marker 100bp with bands from lower to upper of the gel (100-200-300-400-500-600-700-800-900-1000-1200-1500-2000-3000) to the right M Lane Pos: represents the tested positive sample with expected correct size 487 bp

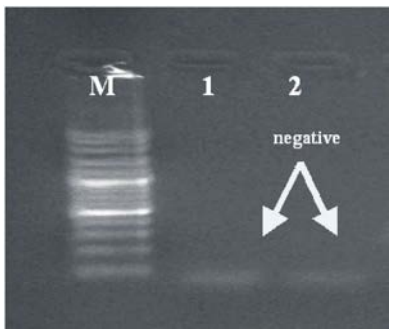

Fig. 4: Ethidium bromide agarose gel electrophoresis of the RT-PCR products of H9N2 and NDV after composting treatment. Lane M: marker 100bp with bands from lower to upper of the gel (100-200-300-400-500-600-700-800-900-1000-1200-1500-2000-3000) to the right M Lane 1: represents the negative RT-PCR product of H9N2 AIV. Lane 2: represents the negative RT-PCR product of NDV

and NDV from mixed respiratory infection in commercial flock farms in Egypt during the last months of 2012 via composting process. Thus we try to evaluate the efficacy of composting process as a method of hygienic disposal of poultry mortalities and their wastes on the presence of AIV H9N2 and NDV mixed infected together in commercial flock farms in Egypt during the last months of 2012 before (Fig. 1, 2 and 3) and after composting (Fig. 4). Determination of nucleotide sequences for the HA gene of the Egyptian strain A/chicken/Egypt/VRLCU-ZK2/2012 was carried out using specific internal primers. Primer sequences were described in Supplementary Table 1. The nucleotide and amino acid sequence analysis was performed for HA gene of the Egyptian strain A/ chicken/ Egypt/VRLCU-ZK2/2012 in comparison with other related strains revealed the closest genetic similarity to the Israeli strains as in (Fig. 6, 7).

Phylogenetic analysis showed that the Egyptian virus, A/chicken/Egypt/VRLCU-ZK2/2012, grouped in the G1/97-like lineage and shared a common ancestor with the G1/97 isolate (Fig.5) revealing the relationships between the HA gene of the recently isolated H9N2 virus from Egypt and other regional and international strains. The HA Phylogenetic tree placed A/chicken/ Egypt/ VRLCU-ZK2/2012 in one group with Israeli strains and other related strains that circulating in the Middle East region (Lebanon, Saudi Arabia, Dubai, Pakistan and China) within the G1/ 97-like lineage.

The identification of The H9N2 subtype in multiple host species combined with its co-circulation with other type A influenza viruses has provided the conditions for H9N2 viruses to accumulate mutations and generate novel variants, thus increasing the probability for this subtype to evolve into a pandemic strain [21]. The avian H9N2 virus possesses human virus-like receptor specificity producing flu-like illness. Therefore virus has the opportunity to circulate and reassort with other influenza viruses [22-24]. The circulation of avian viruses among populations increases the genetic diversity of influenza viruses. AIVs are represented in dozens of antigenic subtypes;17 Haemagglutinin (HA) and 10 Neuraminidase (NA) subtypes [25, 26]. Based on Phylogenetic analysis of the HA gene of H9N2 viruses, there are, so far, two major genetic lineages: the North American and Eurasian lineages [27]. In the latter, several sublineages have been distinguished: The G1-like sublineage was established in the Middle East and on the Indian subcontinent in the 1990s while other sublineages (Y280 and Ck/bei-like) circulate mainly in countries of the Far East [28]. Circulation of H9N2 viruses in the Middle East and Northern Africa since the year 2000 has been frequently reported in Israel, Jordan, Lebanon, Saudi Arabia, the United Arab Emirates, Kuwait, Iraq and Libya, where inactivated vaccines have been implemented in some countries to combat H9N2-associated disease of economical importance in poultry [21, 29, 30]. Usually, poultry infected with H9N2 AIV, unless complicated with other pathogens, show no clinical illness or suffer mild respiratory signs and a drop in egg production [31]. Experimental infection of SPF chickens has shown that H9N2 avian influenza virus is low-pathogenic, but in the last decade, Asian and Middle Eastern countries have faced frequent outbreaks of H9N2 infection with high mortality [31, 32]. Co-infection with other pathogens such

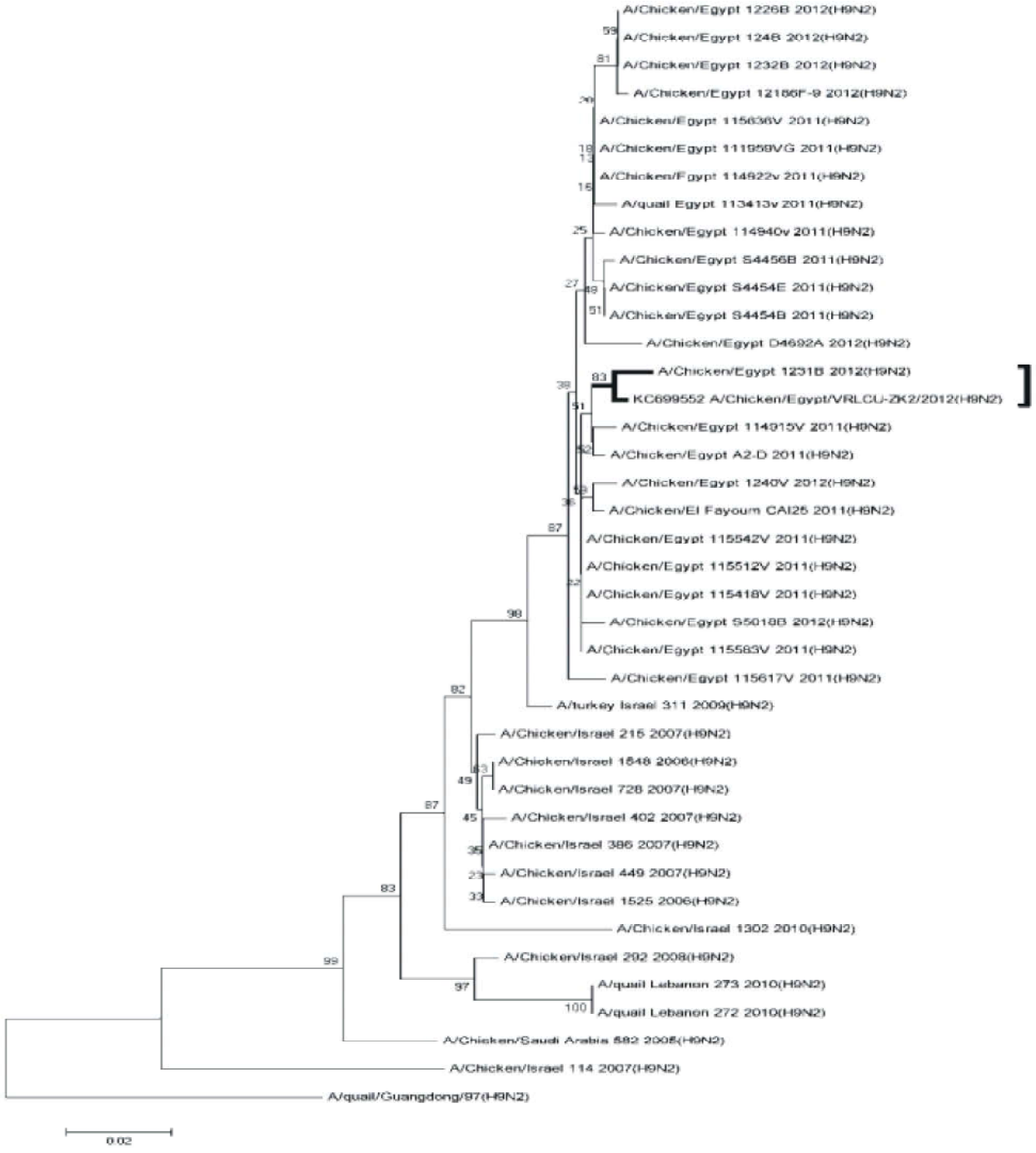

Fig. 5: Neighbor joining Phylogenetic rooted tree based on nucleotide sequence showing the clustering of (A/chicken/Egypt/VRLCU-ZK2/2012 (H9N2) with other representative H9N2 AI strains circulating among chicken population isolated from cecal tonsil. The tree was generated by Mega4 software program

as infectious bronchitis virus can aggravate H9N2 infections resulting in high mortality rates [32, 33]. At the early months of 2011 H9N2 AIV has been reported in the commercial poultry flocks in Egypt causing severe economic losses and mortalities especially when associated with other respiratory diseases (data not shown). Previous unpublished, unofficial reports indicated the presence of H9N2 subtype in Egypt many

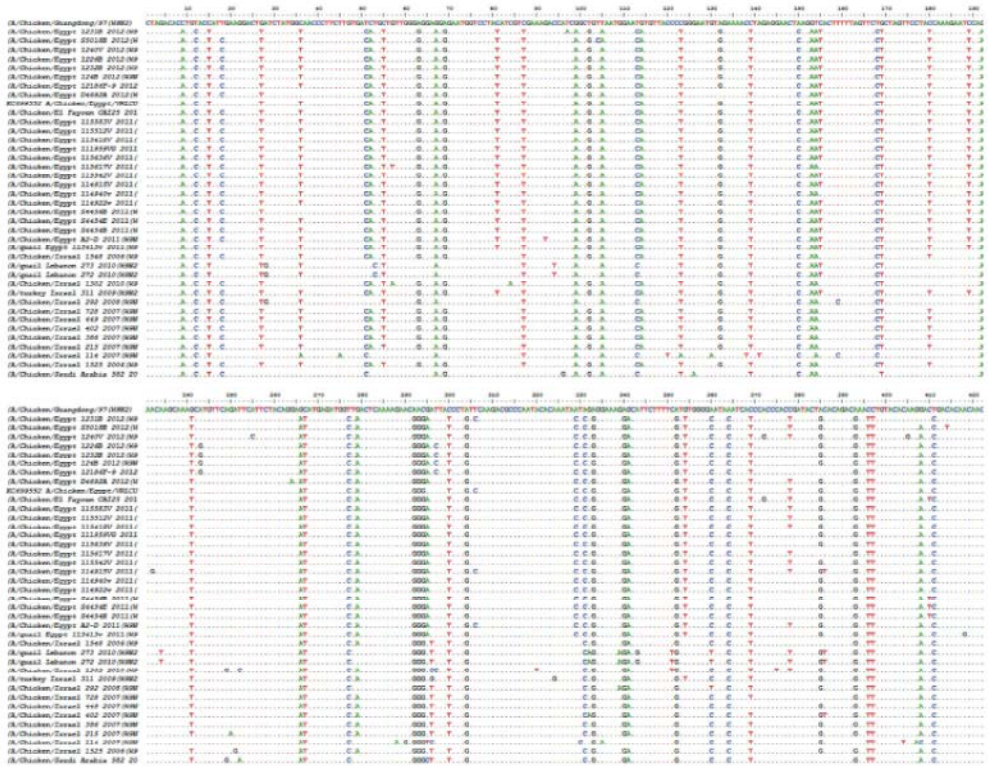

Fig. 6: Multiple nucleotide alignment of of the HA gene of of (A/chicken/Egypt/VRLCU-ZK2/2012(H9N2) partial cds characterized from cecal tonsil in comparison with other H9N2 AI representative strains circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the nucleotide sequence

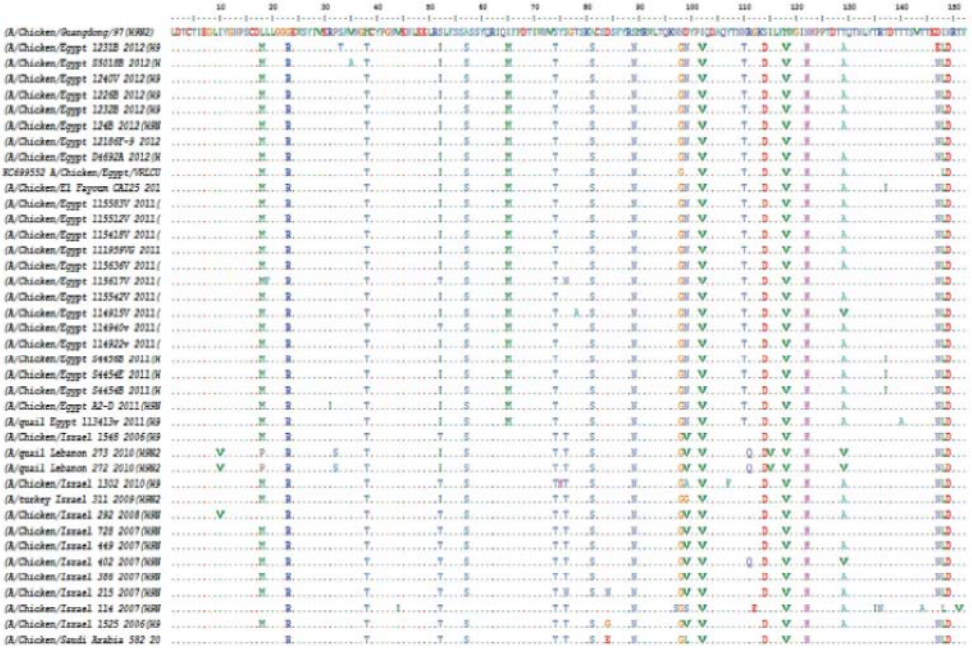

Fig. 7: Amino acid alignment of the HA gene of of (A/chicken/Egypt/VRLCU-ZK2/2012(H9N2) partial cds characterized from cecal tonsil in comparison with other H9N2 AI representative strains circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence

years ago. Furthermore, the H9N2 has already been detected and sequenced from clinical samples collected from live bird markets in Egypt in 2003 and submitted through NAMRU3 to SEPRL, USA to complete virus characterization [34].

CONCLUSION

This article reported the efficacy of composting on the presence of H9N2 AIV and NDV genetic characterization of the genes of an H9N2 AIV isolated from quails in Egypt. Continuous surveillance activities are highly recommended to detect the prevalence of this subtype in commercial poultry in Egypt. Further antigenicity and pathogenicity studies are needed to determine the pathogenic potential of this isolate in avian and animal models since HPAI H5N1 is endemic in poultry in Egypt, a situation that is considered to have potential for the evolution of AIV. The presence of a new subtype of LPAI H9N2 may add another risk factor to the poultry industry in Egypt, especially with the endemic situation of HPAIH5N1 and the presence of other pathogens with low Biosecurity level in some commercial sectors that permit easy virus transmission and adds more stress to the condensed poultry populations.

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