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Efficacy of Composting Poultry Mortality and Farms Wastes with Mixed Respiratory Infection Viruses H5N1 and H9N2 in Egypt

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Abstract: Proper hygienic disposing of dead poultry with respiratory infection and their wastes is imperative from socioeconomic concept. Composting is one of disposing methods and represents a major goal for control and combating this infection. Collected fresh dead birds as well their litter and wastes from broiler farms with high mortalities associated mixed respiratory symptoms were subjected to composting process. The avian influenza viruses (H5N1 & H9N2) were isolated and characterized phenotypically and genotypically from trachea prior subjecting to composting. Compost mix was kept in environmentally controlled composter from 1-28 days (end experiment). Monitoring thermal profile of the composting process was recorded. Failure of re-isolation and characterization of AIV (H5N1 & H9N2) in current work on days 15th confirmed the efficacy of composting poultry farms mortality and wastes with special concern to the current isolated classical AIV H5N1 and H9N2. Secured Composting potentiated microclimatic determinants for both virus strains (heat and dryness) with failure of re- characterization from field dead birds and their wastes. Composting suggested being a reliable, environmentally safe way to dispose poultry mortality and wastes infected with mixed respiratory infection viruses H5N1 and H9N2.

Key words: Composting · Dispose · Mixed infection · H5N1 · H9N2 · Characterization

INTRODUCTION

H9N2 infections of poultry have attracted increasing interest by virtue of the insidious spread of this virus in many Asian countries, together with outbreak reports in the Middle East and concerns about zoonosis and pandemic threat [1, 2]. An outbreak caused by an H9N2 low-pathogenic avian influenza virus (AIV) occurred in a chicken farm and caused severe economic losses. H9N2 virus possesses the capacity to replicate efficiently in the respiratory system against vaccination and to cause severe disease in domestic chickens. Appropriate updating of vaccine strains, based on continuous surveillance data is advised to prevent the possibility of a new H9N2 epidemic in Korea [3]. In Egypt, commercial farms are major reservoirs for influenza (H5N1) virus; the positivity rate was higher for commercial farms (7.2%) than for backyard farms (0.9%). Because the sampled poultry at commercial farms, where biosecurity measures were generally lax, were vaccinated with commercially available subtype H5 vaccines, the effectiveness of such vaccines becomes highly questionable [4]. Influenza A viruses are one of the major threats in modern health care. Novel viruses arise due to antigenic drift and antigenic shift, leading to escape from the immune system and resulting in a serious problem for disease control [5]. The H5N1, H9N2 viruses co-circulate in the poultry population in China. The mRT-PCR assay was established successfully for the detection and differentiation of avian H5 and H9 subtype AIVs [6, 7]. Continuous monitoring of the evolving H5N1 virus in Egypt is essential to develop new control campaigns in poultry and human population. [8]. The extensive circulation of Highly Pathogenic (HP) H5N1 Avian Influenza in Egypt in poultry since 2006 resulted in the emergence of distinct clades with the recent identification of a further clade: 2.2.1.1. Egyptian

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H5N1 viruses have undergone significant antigenic diversification between 2007 and 2010 and two distinct antigenic clusters co-circulated in 2010. Antigenic diversity of H5N1 HP Egyptian viruses may represent a potential challenge for the development of an effective vaccination programme for animal and human health in Egypt [9]. The nucleotide and amino acid sequence analysis of the hemagglutinin gene of the characterized Egyptian H9N2 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) on day 15th of composting treatment confirmed the efficacy of composting poultry farm mortalities and wastes [10]. It is important to note that the practice of accumulating poultry litter (which is mostly used as manure meant for crop farming or sold to crop farmers) is common among the poultry farms in Kano State [11]. Composting have proven to be an environmentally sound method for disposing dead bird. The composting process management and monitoring it´s thermal profile, moisture content, nutrient ingredients were determined and recorded during day 1 to 33th and proved inactivation of H5N1 AIV from poultry mortality and farm wastes due to increased temperature 40-60°C during days5-15th [12-14].

The current experiment was conducted to evaluate the efficacy of composting process as a method of hygienic disposing of poultry mortality and their wastes with mixed respiratory infection on the surviving of isolated AIV H5N1 and H9N2 in commercial broiler farms mortalities in different localities in Egypt late 2012.

MATERIALS AND METHODS

Source and Collection of Specimens: During the last months of 2012, a total of 10 commercial chicken flocks with high mortality around Sharkya and Qalyoubia Provinces were visited and detailed history of the flocks were observed. Those flocks were suffered from respiratory manifestations. The postmortem cross lesions were severe congestion of trachea with mucopurelant exudates, pericarditis and intestinal congestion. Samples were collected from freshly dead and morbid birds. in sterile PBS and transported in cooler to laboratory and kept in freezer at -70°C [15], until processed for molecular diagnosis [16].

Collected poultry farms wastes (built-up litter with dropping, waste food, feathers, detached tissues and droppings) were collected from investigated farms. 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 [17]. The aseptically collected waste samples were sent to Virology laboratory (Faculty of Veterinary Medicine, Cairo University) for detection and confirmation of presence or absence of H5N1 and H9N2 viruses before and after composting.

Laboratory Investigations: Random samples 500-g of the compost mixes were removed from days 1 to 28th according to thermal phases (temperature increased up to 40-60°C).

Monitoring Composting Process: Daily monitoring of the temperature °C and relative humidity % were carried out as described by Ahmed *et al.* [14].

Molecular Characterization of H5N1 and H9N2 AIVs

Viral RNA Extraction: Viral RNA was extracted from a pool of tracheal 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 [18]. All the procedures carried out as recommended by the kit instruction manual.

Primers Design and RT-PCR Reaction: RT-PCR reaction was performed separately for each gene using Promega Access RT-PCR System in a 50 ul reaction as described by Chaharaein *et al*. [19]. Specific primers targeting the matrix (M) gene, H5 gene and H9 gene of AIVs were used (Table 1). The RT-PCR reaction tubes were incubated in the thermal cycler (GeneAmp® 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, 51 °C for H5 and 50 °C for H9 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 both H5 & H9 AIV were used for sequencing.

Table 1: Primer sets to amplify type-specific (M) and subtype-specific (H5 and H9) genes of type A influenza viruses

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	
H5F	GAT TGT AGT GTA GCY GGA TGG	406bp
H5R	CTT GTC TGC TCT KCM KCA TC	
H9F	CTY CAC ACA GAR CAC AAT GG	487bp
H9R	GTC ACA CTT GTT GTT GTR TC	

Matrix (M) gene specific primers and H9 subtyping specific primers were designated by VRLCU (Virology Lab of Cairo University)

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). BioEdit software version 7.0.9.0 [20] was used to analyze and edit the generated sequences of the H5 and H9 genes. The GenBank database was screened (BLAST) for closely related sequences. A phylogenetic analysis of the newly obtained nucleotide sequences Using MEGA 4.0.2, the phylogenetic tree was constructed by the neighbour-joining method and the reliability of each tree branch was estimated by performing 1000 bootstrap replicates [21]. The sequences were submitted to GenBank under the following accession numbers: KC625532.1, KC699552.1.

RESULTS AND DISCUSSION

Positive isolation and characterization of AIVs was recorded from dead and morbid bird's trachea before subjected to composting (Fig. 1). Current positive isolation, characterization and sequence analysis of fragment 4 of H5 gene revealed clustering of the virus with those field strains circulating among chicken population in Egypt in 2011. Previous studies on H5N1 revealed that, despite intense global efforts to arrest the spread of HPAIV H5N1 and to eradicate the virus where it surfaced, the infection established endemic status in poultry populations in several regions including Indonesia and Egypt [22]. Accumulated manure might serve as a source for AI H5N1 viruses. Given that large quantities of AI H5N1 viruses are shed in the feces of infected poultry [23].

The phylogenic tree of H5 gene confirmed the clustering of the characterized virus with the field strains and H5N1 circulation among chicken population in Egypt. The virus circulation previously studied in Egypt by Hafez *et al*. [24], despite the nationwide vaccination, strategy of poultry in Egypt to combat H5N1 AIV,

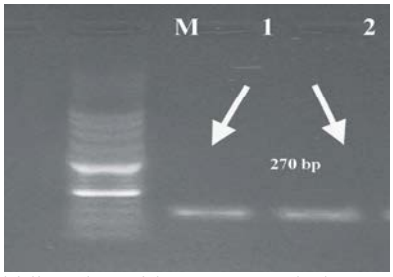

Fig. 1: Ethidium bromide agarose gel electrophoresis of the RT-PCR products of M gene of AIVs before composting. Lane M: marker 100bp with bands from lower to upper of the gel (100-200-300-400-500-600-700-800-900-1000-1200-1500-2000-3000) to the right M Lane 1: represents the negative control. Lane 2: represents the tested positive sample with expected correct size 273 bp

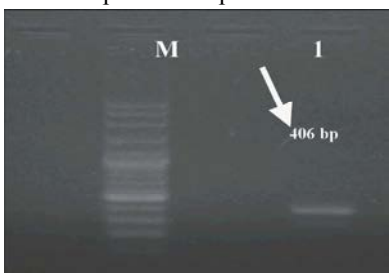

Fig. 2: Ethidium bromide agarose gel electrophoresis of the RT-PCR products of H5N1 AIV before composting. Lane M: marker 100bp with bands from lower to upper of the gel (100-200-300-400-500-600-700-800-900-1000-1200-1500-2000-3000) to the right M Lane 1: represents the positive sample with expected correct size 766 bp. Lane 2: represents the tested positive sample with expected correct size 406 bp

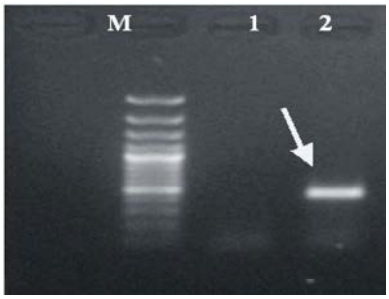

Fig. 3: Ethidium bromide agarose gel electrophoresis of the RT-PCR products of H9N2 AIV before composting. Lane M: marker 100bp with bands from lower to upper of the gel (100-200-300-400-500-600-700-800-900-1000-1200-1500-2000-3000) to the right M Lane 1: represents the negative control. Lane 2: represents the tested positive sample with expected correct size 487 bp

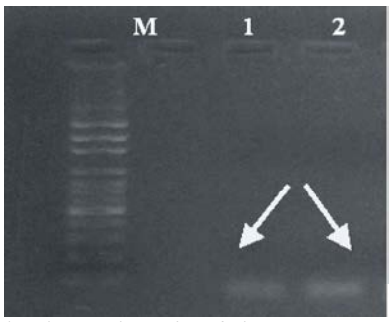

Fig. 4: Ethidium bromide agarose gel electrophoresis of the RT-PCR products of H9N2 and H5N1 AIVs after composting treatment. Lane M: marker 100bp with bands from lower to upper of the gel (100-200-300-400-500-600-700-800-900-1000-1200-1500-2000-3000) to the right M Lane 1: represents the negative RT-PCR product of H9N2 AIV. Lane 2: represents the negative RT-PCR product of H5N1 AIV

Fig. 5: Neighbor joining Phylogenetic unrooted tree based on nucleotide sequence showing the clustering of (A/chicken/Egypt/ZK/2012(H5N1) with other representative H5N1 AI strains circulating among chicken population. The tree was generated by Mega4 software program

Fig. 6: Amino acid alignment of the HA1 gene of (A/chicken/Egypt/ZK/2012(H5N1) in comparison with other H5N1 representative strains of circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence

continuous circulation of the virus in vaccinated commercial and backyard poultry was reported and the efficacy of the vaccination using a challenge model with the current circulating field virus should be revised. Beato *et al*. [9] recorded the antigenic cartography demonstrated for the first time that Egyptian H5N1HPAI viruses can be intuitively separated into two major antigenic clusters EG-antigen-A and EG-antigen-B, which corresponded to clade 2.2.1 and 2.2.1.1 respectively. The efficacy composting birds infected with virus H5 gen with their litter and manure on the virus survival was previously studied by Lu, *et al*. [25] where virus was mixed with manure; this virus was inactivated in 15 min at 56°C, 23 days at 15-20°C and survived even longer at refrigeration temperatures. Neighbor joining Phylogenetic un-rooted tree (Fig. 5), showing the clustering of (A/chicken/Egypt/ZK/2012(H5N1) with other representative H5N1 AI strains circulating among chicken population. The tree was generated by Mega4 software program. Amino acid alignment of the HA1 gene of (A/chicken/Egypt/ZK/2012(H5N1) (Fig. 6) in comparison with other H5N1 representative strains of circulating among chicken population.

H9N2 was recognized and characterized from bird trachea before composting (Fig. 3), where Lane 2: represents the tested positive sample with expected correct size 487 bp. The Phylogenic tree of H9N2 isolated from trachea and currently used vaccines from the Genbank database with accession number (KC699553 441 bp) Egypt /VRLCU-ZK2/2012(H9N2). Neighbor joining Phylogenetic rooted tree (Fig7) showing the clustering of (A/chicken/Egypt/VRLCU-ZK2/2012 (H9N2) with other representative H9N2 AI strains. The identified isolate was originated from circulating Israel turkey's isolates 2009

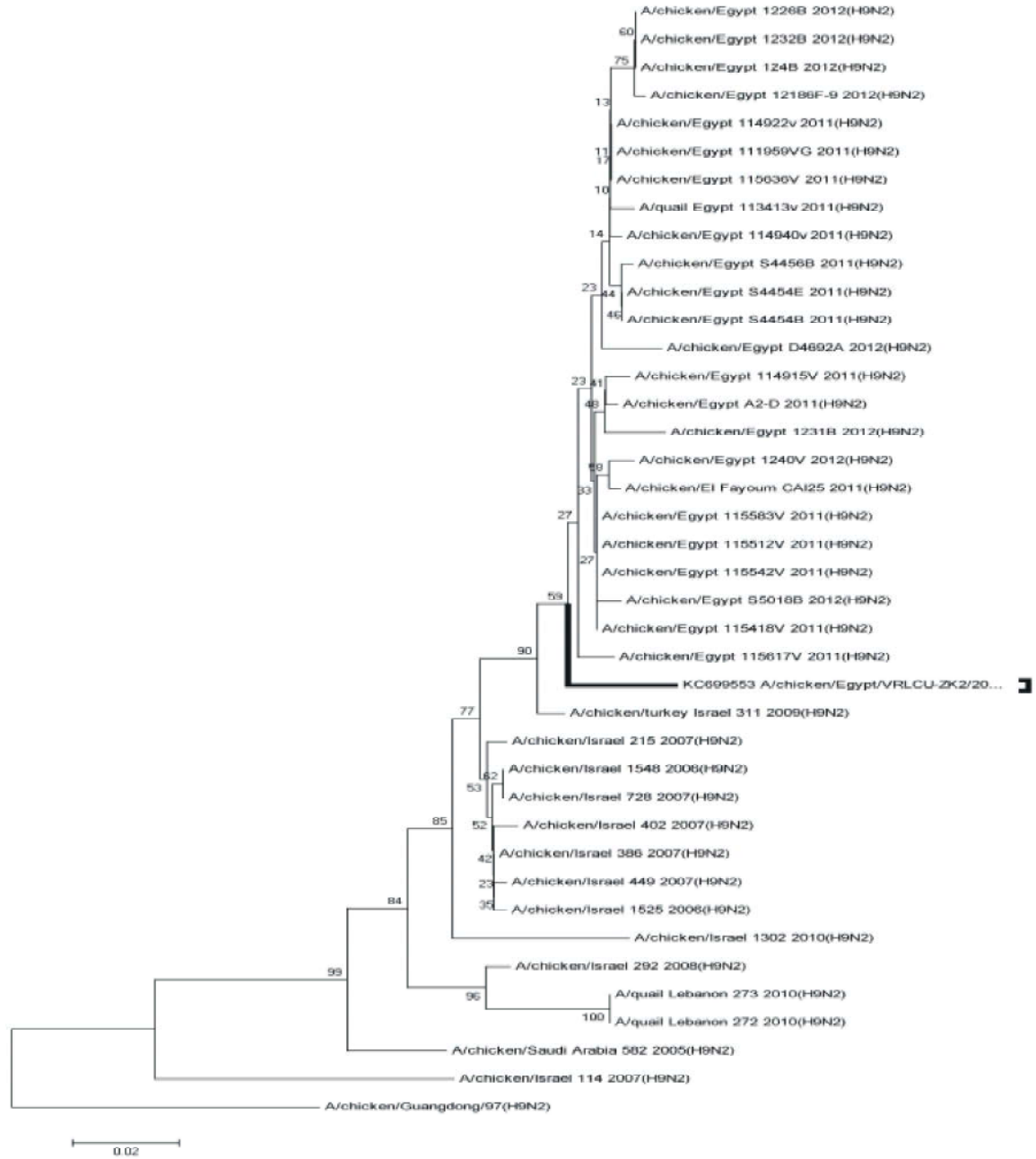

Fig. 7: Neighbor joining Phylogenetic rooted tree based on nucleotide sequence showing the clustering of (A/chicken/Egypt/VRLCU-ZK2/2012 (H9N2) with other representative H9N2 AI strains circulating among chicken population. The tree was generated by Mega4 software program

which previously recognized from Israel chicken 2007 and quail Lebanon 2010 circulating among chicken population. These results may be attributed to the rapid antigenic evolution of H9N2 AIV and resulting antigenic difference from the earlier vaccine strains. The presence of avian and human type receptors in turkey's trachea along with their higher susceptibility to wild and domestic bird origin viruses strengthens the argument that turkeys, like chickens and quail can be potential intermediate hosts for interspecies transmission and spread of reassortant viruses between birds and humans [26]. Japanese quail may provide an optimal environment for the adaptation of wild bird AIV, generating novel variants that can cross the species barrier [27]. Reassortment between influenza

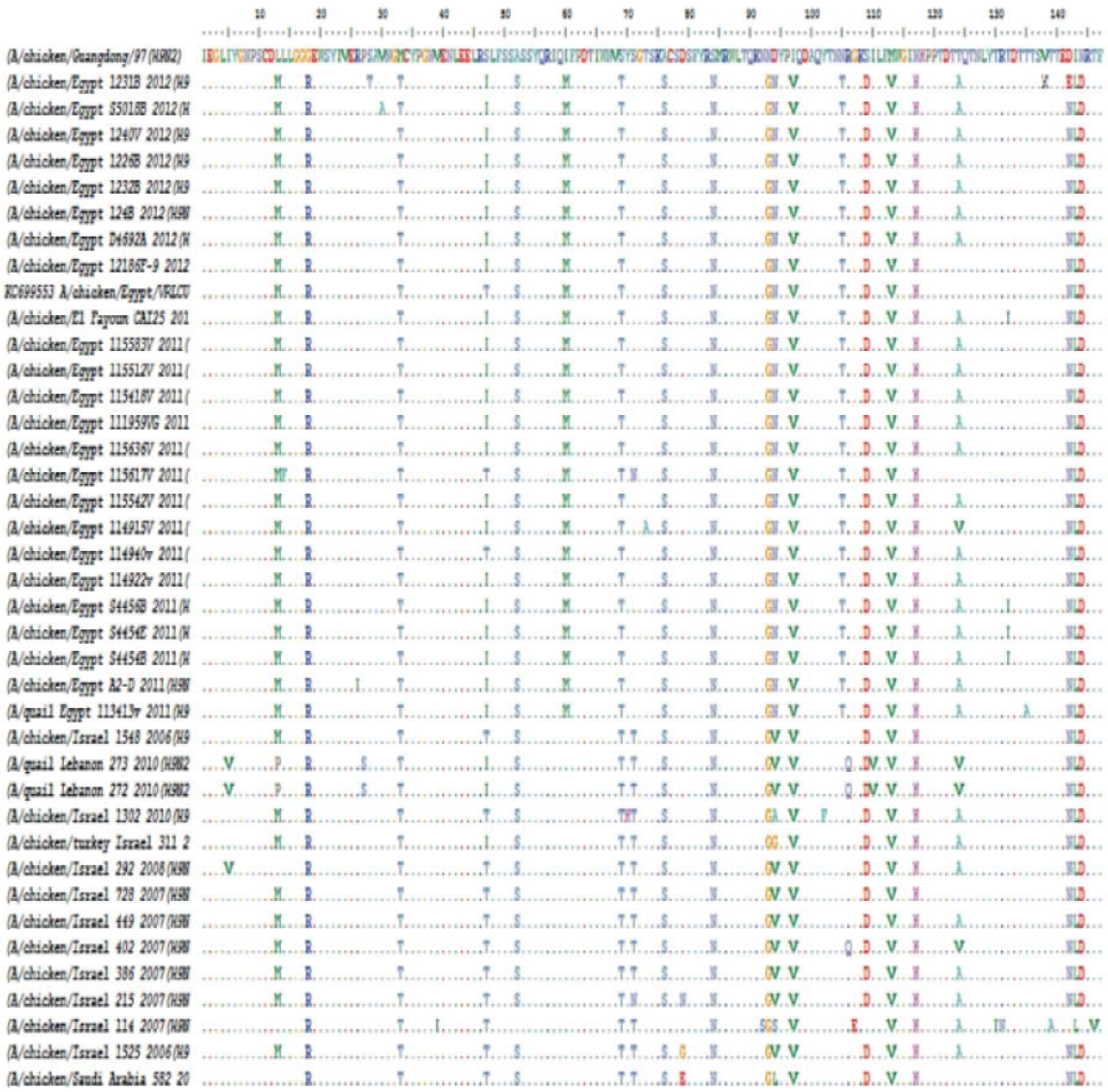

Fig. 8: Amino acid alignment the HA1 gene of of (A/chicken/Egypt/VRLCU-ZK2/2012(H9N2) in comparison with other H9N2 AI representative strains circulating among chicken population. The Dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence

H9N2 and H5N1 in poultry have been reported by Xu *et al*. [28] which may be due to inefficiency of vaccination program in control and combat H9N2 AIV with possibility of inducing respiratory disease with economic losses according to Zhang *et al*. [29], they also advised vaccine strain should be updated in a timely manner through surveillance and accompanying laboratory evaluation of contemporary viruses for antigenic similarity with existing vaccine strains. The avian influenza vaccination policy needs to be re-assessed and increased veterinary Biosecurity on farms rather than vaccine application alone, should be implemented to prevent and control avian influenza according to Sun, *et al*. [30]. Japanese quail may provide an optimal environment for the adaptation of wild bird AIV, generating novel variants that can cross the species barrier [27].

Failure of re –characterization of both isolates (H9N2 & H5N2) after composting (Fig. 4), may be attributed to increased temperature (40-60°C) during composting. Failure of re-isolation of H5N1, H9N2 in current work on day 15th confirmed the efficacy of composting poultry farms mortality and wastes with special concern with the current classical AIV H5N1 as

well with H5N1 variant strains previously accomplished by Zakia *et al.* [14] and for H9N2 previously recorded by Zakia *et al.* [10].

CONCLUSION

H9N2 was recognized and characterized from bird trachea before composting with expected correct size 487 bp and failed to be re-characterized post composting. Neighbor joining Phylogenetic rooted tree showing the clustering of (A/chicken/Egypt/VRLCU-ZK2/2012 (H9N2) with other representative H9N2 AI strains. The identified isolate was originated from circulating Israel turkey's isolates 2009 which previously recognized from Israel chicken 2007 and quail Lebanon 2010 circulating among chicken population. The rapid antigenic evolution of H9N2 AIV and resulting antigenic difference from the earlier vaccine strains requires re-assessing avian influenza vaccination policy and increased veterinary Biosecurity on farms including hygienic disposing of poultry mortality with respiratory infection by composting, rather than vaccine application alone.

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