American-Eurasian J. Agric. & Environ. Sci., 12 (5): 588-596, 2012
ISSN 1818-6769

Efficacy of Composting Dead Poultry and Farms Wastes Infected with Avian Influenza Virus H5N1

[1]Zakia AM Ahmed, [2]H.A. Hussin, [2]M.A. Rohaim and [1]Shimaa Abo El Soud Nasr

[1]Department of Animal Hygiene and Management, Faculty of Veterinary Medicine, Giza, Egypt
[2]Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

Abstract: Composting had proven to be an environmentally sound method for disposing dead birds. The composting process management and monitoring it´s thermal profile, moisture content, nutrient ingredients were determined and recorded during day 1 to 33. Isolation and characterization of H5N1 avian influenza virus (AIV) in freshly dead birds and their wastes before, during and after composting was carried out using RT-PCR based assay and sequence analysis. In composting the temperature was increased gradually from 40-60°C through days 5 to 15 then declined after day 15 till end composting. The dry conditions and increased temperature were important virus determinants. Failure of re-isolation of virus in consequence to increased temperature during composting was proven when tested at day 15, end composting and dryness period. AIV was characterized before composting in the birds trachea and compost mix. 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.Testing the composting mix at the day 15 and end of composting by RT-PCR assay revealed negative amplification confirming the efficacy of composting process for destroying AIV. Composting within the newly designed closed composter achieved unfavorable thermal and dryness conditions for H5N1 surviving with no isolation and characterization of AIV H5N1 from field dead birds and their wastes. The study proposes composting as a reliable, environmentally safe way to dispose poultry waste infected with H5N1 AIV.

Key words: AIV H5N1 · Amino Acids Sequencing · Composting · Ingredients · Surviving Determinants · RT-PCR

INTRODUCTION

The Ministry of Health of Egypt has reported a new confirmed human case of avian influenza A (H5N1) on 27 November 2009 and of the 89 cases confirmed to date in Egypt, 27 have been fatal. [1]. The Ministry of Health of Egypt has announced five new cases of human H5N1 avian influenza infection on 4 March 2010 [2]. 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 (e.g. dead birds, eggs, litter, manure, fresh or frozen carcasses, plant and building materials). Composting is a biological process in which organic wastes are stabilized and converted into a product use as a soil conditioner and organic fertilizer. The process is self-limiting because of excessive accumulation of heat that will eventually fall. Microbial activities require a carbon: nitrogen (C:N) ratio between 15,25 and 30, a moisture content 40 to 60% (w/w), pH between 5 to 12 and from 10- 30% free air space (volume). For poultry waste, a low C/N ratio contributes to large ammonia losses. It was found that 45-60cm of cover material was generally very effective at retaining odorous gasses produced during composting [3,6]. Mortality composting has proven to be an effective, environmentally sound method because rodents, scavenging animals and other pests are seldom a problem, as well; fly larvae, pathogenic bacteria and viruses are destroyed during composting. Moving the material aerates the mixture and revives the microorganisms so another heat cycle can occur, leading to a more complete breakdown of the compost. By use of insulation and turning the compost 3 times during the high-temperature period, it is possible to ensure 5 log reductions in numbers of pathogens. Composting achieves adequate levels of microbial pathogen reduction,

Corresponding Author: Zakia A.M. Ahmed, Department of Animal Hygiene and Management, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.

although spore-forming bacteria and prion agents may not be completely eliminated [7-10]. For composting efficacy on surviving AIV, limited scientific reports were recorded on the safety of poultry mortality composting and even less on the use of this compost on soils used for growing food for human consumption. There have been concerns about prion agents remaining in compost. HPAIV and the adenovirus in composted poultry carcasses were killed by heat (56°C/3 hours, or 60°C/ 30 minutes). After 10 days of composting of chickens inoculated with HPAI virus in bins no virus could be isolated. The loss of infectivity of the AI virus in 15 to 20 minutes at 133°F (56°C) was achieved but it remained viable at moderate temperatures for long periods and indefinitely in frozen material. The most efficient temperature range for composting was between 104-140°F (40-60°C). On-site composting has a high level of biosecurity as no untreated material leave the farm including carcasses and infected litter [11- 13].

In Egypt, clade 2.2 viruses seem to have evolved and diverged significantly compared to other viruses belonging to the same clade in Africa, Europe and Asia as demonstrated by the emergence of a distinct third order clade (namely 2.2.1), which includes all the Egyptian isolates analyzed so far [14, 15].However, it is difficult to determine without further investigations whether this is due to faster evolutionary rate of a distinct population of viruses, or the immune pressure of the vaccination strategy in place [16].

The current experiment was conducted to evaluate the efficacy of composting process as a way of hygienic disposing of dead poultry and their wastes infected with avian influenza virus on the virus surviving in the end compost.

MATERIALS AND METHODS

Field Samples Collection: *Water samples* were collected from main water supplies for broiler farms as well from drinkers inside farms (representative samples from different sites). Sterile containers used for water samples for viral characterization were kept in freezer at -70°C until accomplished.

Freshly dead birds from suspected farms (symptomatically) or previously diagnosed via reference lab for having H5N1 avian influenza by their owners. The samples were collected from different governorates. They were quickly transferred through cooled vehicle and subjected to layering within the previously prepared composting unit. A total of 240 birds with average body weight 1.400 kg was collected from different sources according to availability and farm owner permission. 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 adjusting moisture content as well as relative humidity inside composter.

Freshly dead birds internal organs mainly trachea, lung, heart, spleen and intestinal contents were collected and preserved in sterile PBS and kept in freezer at -70°C.

Collection of bulk poultry waste samples from poultry houses. Random subsamples of poultry wastes from each poultry housing facilities were collected and combined to yield a 0.5m^3 compost bulk sample of each poultry waste source [17].

The aseptically collected samples (500-g) were sent to Biotechnology Laboratory, Faculty of Veterinary Medicine, Cairo University for detection and confirmation of presence or absence of H5N1 avian virus and tracing its origin (phylogenic and sequence analysis) before and after composting.

Laboratory Investigations: Evaluation and characterization of poultry wastes subjected to composting

Random samples 500-g of the compost mixes were removed from days 1 to 35 according to thermal phases (increased up to 40-60°C).

Moisture content was measured for each subsample (50 g) portion to determine dry weight (105°C overnight) (w/w) and ash content (555°C this temperature required for ashing and for hard tissues may reach 800°Cfor 24h [18].

Chemical characterization included analyses of total N and organic carbon (C). Nitrogen was calculated by the Kjeldahl method [19] and carbon was determined as described by Haug [20], total P, potassium (K) by dry ashing and extraction using dilute hydrochloric acid (HCL) [21].

Monitoring Composting Process: Monitoring compost temperatures and maintaining good management practices throughout the entire process helps to ensure the elimination of insect larvae and pathogens in the final product [10]. Daily monitoring of the temperature and relative humidity % were carried out via controlled sensors inserted internally (descended vertically from top toward bottom 60cm for thermal sensor but 30cm for humidity sensors) and readings manifested on LCD.

Characterization of H5N1 Avian Influenza Virus in Dead Birds and Their Wastes Before, During and after Composting Using RT-PCR Based Assay

Extraction of RNA from Samples Using Biozl Reagent (Bio Flux Japan): Total RNA was extracted from the poultry wastes and trachea collected from freshly dead birds using biozol reagent which is ready to use reagent for isolation of RNA from animal and plant tissue or from bacterium.The method followed up the guanidium-acid-phenol extraction method originally developed by Chomcznski and Sacchi [22]. All the procedures were carried out as recommended by the kit instruction manual.

RT-PCR Assay and Sequence Analysis: One-step RT-PCR was performed using the verso one step RT-PCR kit (Thermo, AB gene UK).Twenty five µl RT-PCR mix consisted of 12.5 µl of the 2 X thermo RT-master mix, 1.25 µl enhancer, 0.5 µl enzyme mix and 5µl extracted RNA from the tested samples were added. Primers were used at a final concentration of 20 µM/µl and final volume was reached by adding 3.75 µl nuclease free water.

Reverse transcription was carried out at 50°C for 15 minutes, followed by denaturation step at 95°C for 2 minutes. cDNA was then amplified by 40 cycles of 95°C held for 30 seconds, 49°C for 30 seconds and 72°C for 45 seconds followed by a final extension step at 72°C for 10 minutes. RT-PCR products were visualized by electrophoresis in 1.5% agarose in 1X TAE, ethidium bromide was added to a final concentration of 0.5 µg/ml and the gels were photographed [23]. Primers; CU-H5F (5'-GAC TCA AAT GTC AAG AAC CTT TA-3') and CU-H5R (5'-CCA CTT ATT TCC TCT CTG TTT AG-3') were used to amplify a 189 bp amplicon from HA gene of H5N1 avian influenza virus [24]. One of the strongly PCR products positive samples was sequenced via the service offered by a local agent (lab technology, company) using automatic DNA sequencer in macrogen company, Korea. Sequence analysis was carried out using bioEdit software and the phylogenetic tree was constructed using mega 5 software.

RESULTS AND DISCUSSION

In Egypt, within investigated farms the used main water source was tap water (chlorinated water) which might be contributed to failed isolation of virus H5 gen from main water supplies for farms and drinkers inside farms.

Absence of avian influenza virus from water sources either main or drinkers might be attributed to characters of tap water used as recorded by Brown *et al*. [24] during their rrecent experiments with two HPAI H5N1 strains and eight LPAI viruses. They demonstrated that persistence in distilled water was significantly related to salinity and temperature. They added, under most conditions the H5N1 strains did not remain infective for as long as the LPAI strains, but estimated survival was as long as 158-182 days at 17°C and 28 days at 28°C. Beside, natural waters would also contain organic matter and microbes which may also affect survival. Some data on survival of AI viruses in surface waters collected in Bulgaria indicated that AI viruses do not persist long in water containing microorganisms [25].

Evaluation and Characterization of Poultry Wastes Subjected to Composting: Figure 1 shows that, the thermal inactivation of the avian influenza H5 gene via composting was achieved at 15 days sample mix and continued thereafter till end composting and dryness period. The temperature increased gradually ≤ 40°C until 60°Cbetween days 5 to15. The current thermal inactivation was accomplished within 10 days and agrees with the previously recorded data by Senne [12] where they inoculated chickens with HPAI virus and composted in bins and reported that no virus is isolated after 10 days of composting. In addition, the most efficient temperature range for composting was between 104-140°F (40-60°C) to destroy the AI virus in a short period according to results of Bonhotal *et al.* [13].The relative humidity inside current composting unit decreased from 77-65% (Table 1 and Fig. 1), the current measure was higher than that mentioned by De-Graft-Hanson [26] and Wikinson [6] which was 50-60% (w/w) moisture content.

Figure 2 reveals, moisture content percentage of the composting ingredient before composting was 24.14 for built up litter and 15.89% for new straw, while the end compost product was 22.34%. The Carbon: Nitrogen ratio ranged 21.54-24.33%. The dry conditions of the end compost is one of the virus surviving determinants, as well C:N ratio which coincides with results previously reported by Finstein and Morris [3], Willson [4] and Rynk *et al.* [5]. The obtained end compost free of virus from the newly designed composter was used for agronomic purpose for cultivating edible vegetables (plant watercress and mint) in agronomic ratio 1:2, compost: soil. This composting process confirmed the more secure and environmental safe level which coincides with Speir and Ritz [27] when they collected and treated all dead birds carcasses in a well designated and functioning composter, the rank of this process is considered low risk (rank 4). However, extremes of heat, dry conditions and extremes of pH do not favor virus's survival. The viruses had been

Table 1: Thermo-Hygro Profile of Composting Process

	Composting Conditions	
Days of Sampling	Temp.°C	R.H %
1	29	70
3	38	74
5	40	77
7	42	72
9	45	69
11	49	72
13	57	75
15	60	65
20	45	62
25	40	60

Table 2 -a: Groups of samples and RT.PCR results before, during and after composting

Gp1	RT.PCR for GP1	Gp2	Gp3	Gp4	RT.PCR for GP1-GP4
1-Trachea	No virus Detection	1-Intestinal Content	1-Spleen	1-Drinkers	No virus detection
2-Trachea	Positive virus detection of H5 gene	2-Spleen	2-Liver	2-Drinkers	
		3-Heart	3-Intestinal Content	3-Drinkers	
			4-New litter	4-Drinkers	
			5-Built-up litter	5-Main water	
			6-Main water		
			7-Main water		
			8-Main water		

*Water samples are collected from different farms and pooled within same farm
*Organs samples are collected and pooled also from same farm (5 individual for each 1000 bird if farm capacity 5000, but when more, one bird / 1000 capacity).

Table 2-b:

Gp 5 Before Composting	Time	Gp.6 during and After Composting	Time of sampling	RT.PCR for detection of
H5 gene for GP5,GP6				
1-New Litter	1d	1-Surface Layer 30cm	17d	No virus detection
2-New Litter	1d	2-Deep layer 70cm	17d	No virus detection
3-Built-up	1d	3-Top compost 30cm	28d	No virus detection
4-Built up Litter	1d	4-Deep compost 70,100cm	28d	No virus detection
5-Compost	During composting 15d	5-Dried end compost	33d	No virus detection

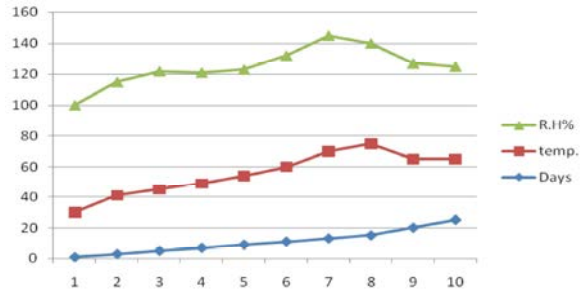


Fig. 1: Thermo-hygro profile of composting process. Days of sampling : 1, 3, 5, 7, 9, 11, 13, 15, 20 and 25 day

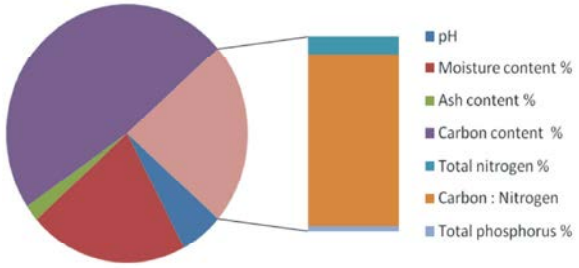
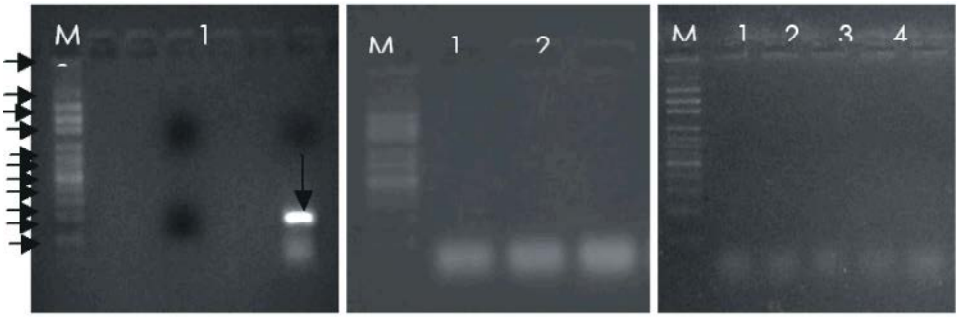


Fig. 2: Chemical analysis of poultry wastes just prior, during and after composting.

known to survive for long periods in manure and litter so composting or stacking to generate heat will be effective in killing them according to De-Graft-Hanson [26].

Table 2 a, b and Figure 3 show the RT-PCR results on the tested samples, revealed the presence of H5 gene in some of birds lungs (pooled samples from some of Giza farms) before composting which disappeared post composting. Results confirmed the effectiveness of composting process done in this study. The efficacy of adding birds infected with virus H5 gen with their litter and manure on the virus survival was previously studied by Lu *et al*. [28] where virus was mixed with manure, inactivated in 15 min at 56°C, 23 days at 15-20°C and survived even longer at refrigeration temperatures. On the other hand, experiments conducted in Thailand [29] demonstrated that H5N1 virus mixed with dry manure was



GP 1 GP 2 GP 3

*Gp1: Vertical arrow directed to trachea no. 2 revealed postive detection of H5 gen. It was included all pooled trachea in five examined samples before composing.The horizontal arrows directed toward left side of Gp1 legend started from down-upward as follow 100,200,300,400,500,600,700 ,800,900,1000,1100bp. The same data was denoted for all legends till 3000bp.

*Gp2: Collected pooled samples were from three organs included intestinal contents, spleen , heart

*Gp3: Collected pooled samples were from three organs spleen, liver, intestinal content and new litter, built-up litter.

GP 4 GP 5 GP 6

*Gp.4: Water samples were collected from main sources and drinkers.

*Gp.5: New and built-up litter samples were used in layering before composting on first day of preparing the composter, while the fifth sample was collected at day 15 of compositing process.

*Gp.6: Samples were collected on day 17 from composter, one from top depth 30cm and the second form top depth 70cm. On day 28 samples were collected on top depth 30, 70, 100cm from the compost mix.

*Molecular weight of marker 100bp (100-200-300-400-500-600-700-800-900-1000-1500-2000-3000).

*All figures of groups except GP1 show negative results which indicated by primer dimer only without any specific band size while in GP1 shows specific band size at 189 bp.

inactivated within one day at 25°C and more rapidly at higher temperatures. According to Ellin Doyle *et al*. [30] virus survival appeared to be longer in moist environments but it might be that, as demonstrated in experiments in water above, the HPAI N5N1 virus was not well adapted to survive in the environment as some LPAI viruses.

Characterization of H5N1 avian influenza virus in dead birds and their wastes before, during and after composting using RT-PCR based assay

Figure 4 shows the phylogenic analysis of the obtained sequence of RT-PCR products shown in Figure 5 confirmed the clustering of the characterized virus with the field strains and H5N1 circulation among chicken population in Egypt. The virus circulation was previously studied in Egypt by Hafez *et al.* [31] where they reported that, despite the nationwide vaccination, strategy of poultry in Egypt to combat H5N1 AIV, 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. In current study, the obtained results were from Gene bank titled (Influenza A virus (A\ chicken\ Egypt\VRL CU\2011

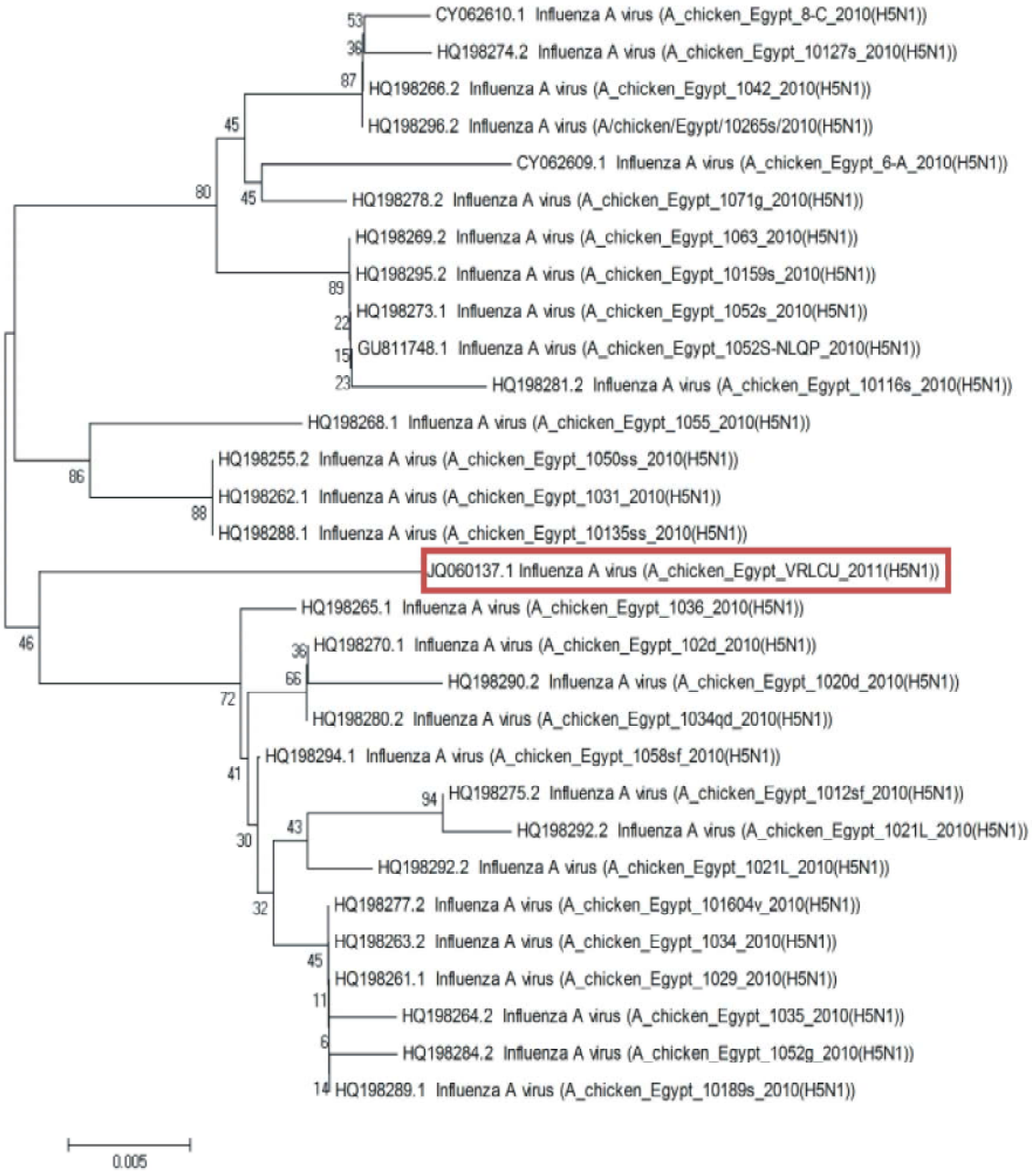


Fig. 4: Phylogenetic tree of the obtained sequence of virus H5 gene isolated from trachea of birds collected from poultry Giza farms in relation with the Egyptian field strains of H5N1 circulating among chicken population in Egypt .The tree was generated by mega5software by macrogen Company, Korea.

(H5N1) Segmente 4 hemagglutinin (HA) gene, partial CDs. Genebank JQ60137.1. The circulation of H5N1 among chicken population might be attributed to absence of routine biosecurity procedures, which were observed in most of investigated poultry farms in this study. The current results revealed no major amino acid changes in such isolated strain when compared with the recent characterized in 2011 in Egypt proposing that the characterized virus was evolved from viruses circulating among the chicken population in the absence of biosecurity measures.

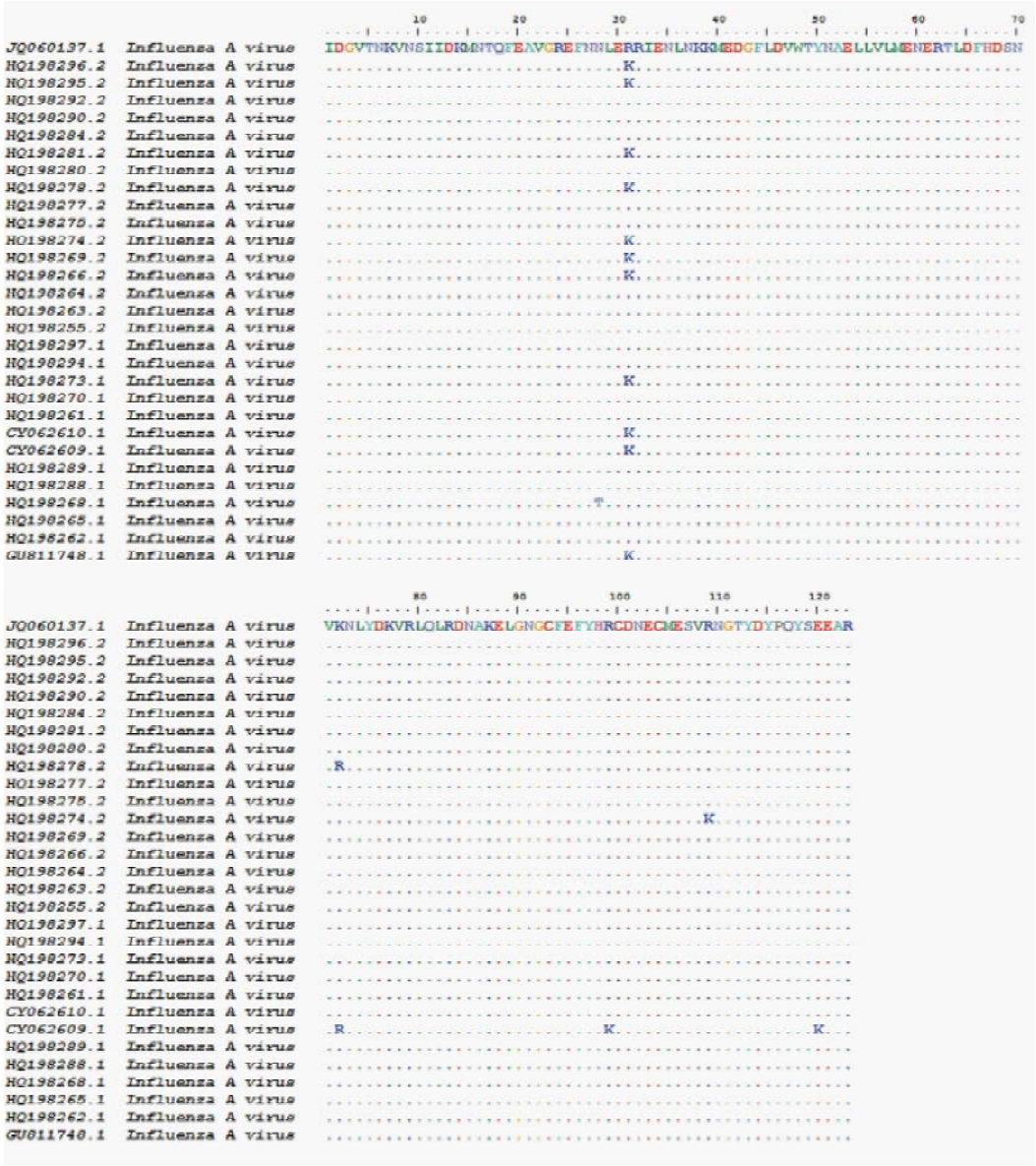


Fig. 5: Sequence analysis of one of positive samples to confirm the presence of H5 gene in the samples before composting .Sequence analysis was carried out using bioEdit software by macrogen Company, Korea..Amino-acid sequence of the obtained fragment in companion with the Egyptian field strain circulating among chicken population denoted with colored letters.

Despite sequence analysis of the recently isolated AIV strains from vaccinated flocks in Egypt showed the occurrence of mutations in critical sites as recorded by. Hussein *et al.* [23], three years on, the impact on disease control of AI vaccination in Egypt has been very limited. A recent assessment study highlighted substantial weaknesses in the current immunization programme and its lack of positive impact on the spread of infection or the maintenance of public health safety. Without strict bio-security precautions undertaken during its application, farm visits to vaccinate poultry could facilitate the spread of the virus and therefore become a risk factor with important implications on the maintenance of the virus and potential risk for human exposure [32].

This experiment confirms one of the most important tools for AIV combating strategy via hygienic disposing of dead birds and their belongs infected with this virus by composting.

ACKNOWLEDGMENT

Financial support for this work was provided by the Cairo University through 5th International Conference of Scientific Research and Its Application FICSRA-3/7, 2009-2011. Authors indeed appreciated efforts and guide of Prof. Dr. Hasanein, F.M and Prof. Dr. Hamoud. M.M., dept. Animal Hygiene and Management and Biotechnolgy Center, Faculty of Veterinary Medicine, Cairo University for support and facilities presented to teamwork of this project.

CONCLUSION

Using a newly designed closed composter unit ensured dry conditions and increased temperature gradually from day 5 to 15 which were important virus determinants. Failure of re-isolation of virus in consequence to increased temperature during composting was proven when tested at day 15, end composting and dryness period. The study proposed composting as more reliable and environmentally high safe method for disposing dead birds infected with AIV H5N1.

REFERENCES

1. WHO, World Health Organization, 2009. Avian influenza-situation in Egypt-update Available:http://www.who.int/csr/don/2009_11_27/en/index.html.
2. WHO, World Health Organization, 2010. Avian influenza-situation in Vietnam-update 8 Available: http://www.who.int/csr/don/2010_03_04a/en/index.html
3. Finstein, M.S. and M.L. Morris, 1975. Microbiology of municipal solid waste composting. Adv. Appl. Microbiol., 19: 113-151.
4. Willson, G.B., 1989. Combining raw materials for composting. Biocycle, August, pp: 82-85.
5. Rynk, R., M. Kamp, G. Willson, M. Singley, T. Richard, J. Klega and F. Gouin, 1991. In: On-Farm Composting Handbook. Northeast Regional Agricultural Engineering Service, 152 Riley-Robb Hall, Cooperative Extension, Ithaca, NY 14853-5701, USA, pp: 174.
6. Wilkinson, K.G., 2011. On-Farm Composting of Dead Stock. In: Integrated Waste Management-II.1st edition. pp: 269-284.Edited by Sunil Kumar:
7. Kalbasi, A., S. Mukhtar, S.E. Hawkins and B.W. Auvermann, 2005. Carcass composting for managing farm mortalities: A review. Compost Science and Utilization, 13: 180-193.
8. Keener, H. and D. Elwell, 2006. Mortality composting principles and operation, In: Ohio livestock mortality composting manual, 1-7, Ohio State University Extension.
9. Berge, A.C.B., D. Thomas Glanville, P.D. Millner and D.J. Klingborg, 2009. Methods and microbial risks associated with composting of animal carcasses in the United States. JAVMA, 234, 1, Vet Med Today.
10. Ritz, C.W. and J.W. Worley, 2009. Poultry mortality composting management guide. In: Composting Poultry Mortalities. Eds., Merka, B., M. Lacy, S. Savage, L. Vest and C. Hammond, Circular, pp: 819-15. University of Georgia Cooperative Extension, Athens, Ga.
11. Senne, D.A., B. Panigrahy and R.L. Morgan, 1994. Effect of composting poultry carcasses on survival of exotic avian viruses: highly pathogenic avian influenza (HPAI) and adenovirus of egg drop syndrome. Avian Dis., 38: 733-737.
12. Senne, D.A., 2007. Avian Influenza in North and South America, 2002-2005. Avian Diseases, 51: 167-173.
13. Bonhotal, J., M. Schwarz and N. Brown, 2008. Natural Rendering: Composting Poultry Mortality The Emergency Response to Disease Control. Cornell Waste Management Institute. Department of Crop and Soil Sciences.
14. Cattoli, G., I. Monne, A Fusaro, *et al.,* 2009. Highly pathogenic avian influenza virus subtype H5N1 in Africa: a comprehensive phylogenetic analysis and molecular characterization of isolates. PloS One, 4, e4842.
15. WHO/OIE/FAO H5N1 Evolution Working Group. 2009. Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: divergence of clade 2.2 viruses. Influenza and Other Respiratory Viruses, 3: 59-62.
16. Peyre1, M., H. Samah., Y.J. Makonnen., A. Saad, Amira Abd-Elnabi, S. Galal, T. Ettel, G. Dauphin, J. Lubroth, F. Roger and J. Domenech, 2009. Minireview. Avian influenza vaccination in Egypt: Limitations of the current strategy. J. Mol. Genet. Med., 3: 198-204.

17. Wood, C.W., M.C. Duqueza and B.H. Wood, 2010. Evaluation of Nitrogen Bioavailability Predictors for Poultry Wastes. The Open Agriculture Journal, 4: 17-22.
18. Atkinson, C., D.D. Jones and J.J. Guathier, 1998. Biodegradability and microbial activities during composting poultry litter. Poultry Science, 75: 608-617.
19. Bremner, J.M., 1996. Nitrogen-total. In: Sparks DL, editor. Methods of soil analysis. Part 3-Chemical methods. Madison, WI: SSSA Inc., pp: 1085-121.
20. Haug, R.T., 1980. Compost Engineering: Principles and Practice. Ann Arbor Science Publ., Inc., Ann Arbor, MI, pp: 347.
21. Hue, N.V. and C.E. Evans, 1986. Procedures used for soil and plant analysis by the Auburn University Soil Testing Laboratory. Auburn University, AL: Alabama Agric Exp Sta.
22. Chomczynski, P. and N. Sacchi, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.
23. Hussein, H.A., H.A. Sultan, A.H El-Deeb and A.A. El-Sanousi, 2008: Possible causes of re-emerging outbreaks of H5N1 Avian influenza virus in vaccinated chickens in Sharkia governorate in Egypt. Egyptian Virol. 5(1): 74-87.
24. Brown, J.D., D.E. Swayne., R.J. Cooper, R.E. Burns and D.E. Stallknecht, 2007. Persistence of H5 and H7 avian influenza viruses in water. Avian Dis., 51: 285-289.
25. Zarkov, I.S., 2007. Survival of avian influenza viruses in filtered and natural surface waters of different physical and chemical parameters. Rev. Med. Vet., 157: 471-476.
26. DeGraft-Hanson. J., 2011. Avian Influenza: What It Is and How to Protect Against Its Establishment. Extensive services, West Virginia University.
27. Speir, A. and C. Ritz, 2009. Broiler litter management. Farm assessment System. Cooperative Extension. University of Georgia.
28. Lu, H., A.E. Castro, K. Pennick, J. Liu, Q. Yang, P. Dunn, D. Weinstock and D. Henzler, 2003. Survival of avian influenza Virus H7N2 in SPF chickens and their environments. Avian Dis., 47: 1015-1021.
29. Chumpolbanchorn, K., N. Suemanotham, N. Siripara, B. Puyati and K. Chaichoune, 2006. The effect of temperature and UV light on infectivity of avian influenza virus (H5N1, Thai field strain) in chicken fecal manure. S E Asian J. Trop. Med. Pub. Health, 37: 102-105.
30. Ellin Doyle, M., S. Schultz-Cherry, M. Robach and R. Weiss, 2007. FRI BRIEFINGS. Destruction of H5N1 Avian Influenza Virus in Meat and Poultry Products. Food Research Institute, UW-Madison.
31. Hafez, M.H., A. Arafa, E.M. Abdelwhab, A. Selim, S. G. Khoulosy, M.K. Hassan and M.M. Aly, 2010. Avian influenza H5N1 virus infections in vaccinated commercial and backyard poultry in Egypt. Poult. Sci., 89(8): 1609-1613.
32. Rynk, R., M. Van de Kamp, G.B. Willson, M.E. Singley, T.L. Richard, J.J. Kolega, F.R. Gouin, L. Laliberty, Jr, K. Day, D.W. Murphy, H.A.J. Hoitink and W.F. Brinton, 1992. On-Farm Composting Handbook. NRAES, Cornell University, Ithaca, NY. pp: 186.