Detection of Avian Influenza (H5N1) In Some Fish and Shellfish from Different Aquatic Habitats across Some Egyptian Provinces

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Abstract: The global climatic changes impact on air, water and earth could extend scope of Avian Influenza (H5N1) virus to another broad sector of creatures including aquatic animals, especially those with direct relationship to aquatic birds. In the current study, Avian Influenza virus (H5N1) was detected in hemolymph of the Red Swamp crayfish (*Procambrus clarkii*) from three different provinces across the Nile Delta. Most of the positive cases were from the neighborhood of migratory bird natural stop stations. The virus was also detected in the Mediterranean Cone Shell (*Conus mediterraneus*) and the Pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) during its course of invasion to the Mediterranean Sea. Two out of three poultry manure samples collected prior to earthen pond fertilization at three different localities were proved to be positive for the H5N1 virus. Tissue / mucous samples collected from earthen pond raised tilapias were negative for the virus. Catfish (*Clarias gariepinus*) has presented a striking model for aquatic species carrying the virus in their blood. The current results are suggestive for an important epidemiological role played by aquatic animals in spread of avian influenza (H5N1) virus across the Egyptian aquatic habitat.
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1. Introduction

The global surge of relatively large number of epidemics as well as pandemics has had deleterious impacts on world socioeconomics. During the past few decades, several epidemics have drastically influenced animals as well as human health. These conspicuous circumstances quicken the search for safe and secure alternatives to terrestrial animals and perhaps looking in the vast blue areas (water) covering more than 75 % of the globe. Aquaculture, the so called "Blue Revolution" was thought to be the ideal alternative to help get out of the dilemma. Unfortunately, due to interference of many environmental / biological factors that might possibly included global warming, aquaculture integration, maritime trade, globalization, overseas continental migratory bird' flyways; aquatic creatures became another vulnerable possible source for devastating pandemics.

Influenza viruses of avian origin have been implicated in outbreaks of influenza in marine mammals, such as harbor seals. Geraci *et al.*, 1982 have indicated that more than 400 harbor seals, most of them immature, died along the New England coast between December 1979 and October 1980 of an acute pneumonia associated with influenza virus A/Seal/Mass/1/180 (H7N7). The isolated virus has avian characteristics, replicates principally in mammals, and causes mild respiratory disease in experimentally infected seals. In 1986, several influenza epidemics have erupted among the cetaceans 'populations at Atlantic and Pacific oceans (Hinshaw *et al.*, 1986). The nucleoprotein (NP) genes of influenza viruses were sequenced from a variety of virus isolates derived from marine mammals: whales from the Pacific / Atlantic oceans, seal and gull from the Western Atlantic, and a tern from the Caspian Sea (Mandler *et al.*, 1990). In comparison to published NP sequences, they found pairs of NPs derived from avian and marine mammal isolates to be closely related, e.g., the gull-whale and mallard-seal pairs from the Atlantic Coast of the USA and the tern-Pacific Ocean whale pair of the Eastern Hemisphere (Mandler *et al.*, 1990).

Known as integrated livestock-fish farming, the technique involves transferring the wastes from raising pigs, ducks or chickens directly to fish farms, with chicken or duck sheds sometimes located directly over the fish ponds themselves. At the correct amount, the nutrients in the manure give potential stimulus to the growth of plankton in the ponds, which are the predominant food for fish such as carp,

tilapia and mugil. Several authors have emphasized that integrated fish farming is now the main basis for aquaculture in China and many bordering countries (Little and Muir, 1987; Edwards *et al.,* 1988; Phong, 2010). Integrated farming with a fish species provides an efficient usage of on-farm resources, increased food and income opportunities, fewer economic risks through farm diversity, and especially effective disposal of crop residues and animal wastes by recycling these materials into high quality fish protein (Little and Muir, 1987; Edwards *et al.,* 1988; NACA, 1989). While the potential of integrated farming present a promising future for global economy, it should be noted that on-farm practice of integrated crop-livestock-fish farming represents less than one percent of farming populations in the tropical countries (Smith, 1988).

Scholtissek and Naylor (1988) were first to discuss the expected role of integrated fish farming systems in the spread of influenza pandemics. Further, Experimental studies suggest that there might be strong links between the resurgence of influenza A virus epidemics and the integration of aquaculture with duck and pig farming close to human dwellings. The transmission of genetic material from ducks to human influenza viruses seems to occur by reassortment in pigs (Kida *et al.,* 1994; Dong *et al.,* 2011). Pigs can become infected with and may transmit both human and avian influenza viruses not only to other pigs but also to the original avian host (Kida *et al.,* 1994; Furuse *et al.,* 2010). Thus, pigs are thought to be 'mixing vessels' where reassortment between avian and human influenza A viruses occurs, resulting in an antigenic shift that ends with the evolution of new human influenza strains with new surface antigens (Kida *et al.,* 1994; Itoa and Kawaoka, 2000; Furuse *et al.,* 2010). For this specific reason, Scholtissek and Naylor (1988) have recommended the development of integrated aquaculture systems in which pigs are reared in enclosed farms away from ducks.

Throughout the past few decades, poultry manure has been widely used as a major source of organic fertilizer to fish ponds in several nations along the Asian continent (Hu and Zhou, 1989; Subosa, 1992; Knud-Hansen *et al.,* 1993). The main reason for adding animal manures to fish ponds is to provide degradable organic matter, which is the most important component to promote the growth of bacteria (Schroeder, 1980; Hu and Zhou, 1989). During the decomposition of such bacteria, CO_2, phosphorus, nitrogen are liberated to constitute essential constituents for phytoplankton's growth (Schroeder, 1980). Planktons represent the bottom of the food chain for fish. Partially, poultry manure can be directly utilized as a food for several cultured fish species like tilapia, mullet and carp (Phong *et al.,* 2007).

Although the recycling of manure in integrated agriculture-aquaculture farming systems presents numerous benefits, the transmission / spread of diseases to human via aquatic organisms multiplying in excreta-laden water requires special attention. There is strong evidence that aquatic organisms may be more important vectors for human diseases than generally realized. However, conclusive epidemiological studies linking the use of excreta in aquaculture with human diseases are lacking (Naegel, 1990). Great portion of the huge increase in China's recent inland aquaculture production is attributed to organic fertilization, provided by the parallel spectacular growth of poultry and pig production. In the past few years , some global wildlife organizations such as Birdlife International has called for an investigation into the possibility that these thousands of manure-fed ponds across Asia may be the means by which the lethal strain of avian influenza, H5N1, is being spread. Birdlife has declared that outbreak of H5N1 occurred in 2004 / 2005 at diverse locations in China, Romania and Croatia where linked to the locations at which fish ponds are widely distributed (Feare, 2006).

Although the concentration of viruses and bacteria leaching from the manure into the aquaculture system is reduced drastically by dilution, filter feeders (e.g. mullet, shrimps, crayfish and snails) can concentrate these pathogens in its body fluids / surface. Despite the die-off pattern associated with this condition and high dilution in the water, they can propose a possible health hazard (Naegel, 1990). Author has also emphasized that convincing epidemiological studies still have to be done to link the risk of bacterial/viral infections to the consumption of aquatic organisms produced in manure-laden ponds. In recent studies, however, it has been proven that after exceeding a rather clearly defined threshold concentration of pathogens in the water, both viruses and bacteria are able to penetrate into the peritoneal fluid and even into the muscles of fish (Buras *et al.,* 1985; Buras *et al.,* 1987). This has an impact on the transmission of viruses and bacteria to persons who have direct contact with the intra-peritoneal fluid and blood of infected fish, like fish handlers and housewives when they are cutting, gutting and cleaning fish in preparation.

There has been overall agreement between the findings of various influenza surveillance studies in migratory birds in regard to the role played by birds in the emergence of pandemics in humans, lower animals, and domestic poultry (Halvorson *et al.,* 1983). The occurrences of outbreaks of highly pathogenic avian influenza H5N1 in Romania,

Turkey and Croatia in October 2005 have all been close to wetlands (Feare, 2006). This, together with their timing, has implicated the migration of migratory aquatic birds from southern Siberia in bringing the virus to Eastern Europe. In autumn 2004, stacks of poultry waste, including dead birds, were dumped next to fish farms at Varazdin, Northeast Croatia (Feare, 2006). The dead bird's stacks were left to seep into the ponds as fertilizer. In Serbia, manufactured poultry manure fertilizers are added to fish ponds; they are believed to be imported but their origin was not known. The association of some wild bird deaths with proximity to fish farms led to this search for information on fish farming practices that could be involved in avian influenza transmission (Feare, 2006). It is imperative to recognize that virus movement between wild birds and poultry is not a one-way street. For example, wild birds may become infected through feeding on infected poultry carcasses (Kwon *et al.*, 2005), and the practice of fertilizing fish ponds with poultry manure, which is widespread in Asia and Eastern Europe , proposes a route by which a wide range of aquatic birds might become infected (Melville and Shortridge, 2006; Bennum, 2006; Brown, 2006).

Molecular techniques such reverse transcriptase polymerase chain reaction (RT-PCR), multiplex transcriptase PCR and real time PCR are the most accurate / rapid tools for the detection of viral particles in clinical specimen as well as environmental samples (Saberfar *et al.*, 2007; Koehler *et al.*, 2008). RT-PCR can also be utilized as a rapid screening method for the simultaneous detection of type A influenza virus, H5 and H9 subtypes in clinical samples (Saberfar *et al.*, 2007; Ip *et al.*, 2008).

The main goal of the current study is to investigate the possible epidemiological role played by some fish /shellfish including native and invasive species in transmission and spread of the avian influenza (H5N1) virus across the Egyptian aquatic habitat.

2. Materials and Methods

Sampling locations:

Sampling locations selection criteria was based upon previous Egyptian official reports on the detection of the Avian Influenza virus in poultry populations from several provinces scattered through the Nile delta. Thus, our sampling protocol was planned to cover vast areas of freshwater as well as marine aquatic habitats located at the basin and within the core of poultry farms neighborhood at these provinces (Plate 1). Definite sampling sites have included several earthen ponds at Sharkiya (Abassa and Eltel-Elkabeir), Manzala Lake basin at both Dakhleya and Damietta, Port Said (Ashtoum Algami Natural Wilderness) and Alexandria (Abou-Qir Bay) (Plate 1).

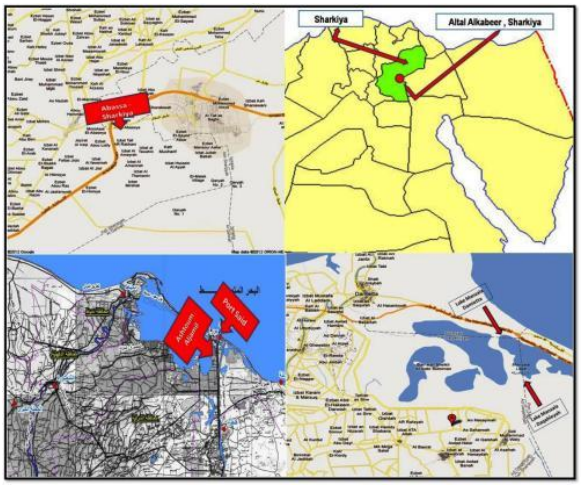

Plate (1). A map showing the geographical locations of fish / shellfish sampling sites.

Sample collection:

Throughout the period from July 2010 till September 2011 a total of 160 live red swamp crayfish (*Procambrus clarkii*), 30 Mediterranean cone shell (*Conus mediterraneus*), 25 Pufferfish (*Lagocephalus sceleratus*), 30 sharp toothed catfish (*Clarias gariepinus*) and 400 live Nile tilapia (*Oreochromis niloticus*) fingerlings, were collected from number of earthen pond based fish farms located at the vicinity of the migration route of some transcontinental migratory aquatic birds crossing the mid-zone of the Egyptian Nile Delta at the above mentioned locations (Table 1) . The endemic nature of avian influenza virus (H5N1) were the triggering factor behind selection of certain delta provinces as sites for wild aquatic bird / poultry manure sampling . Cloacal swabs from a total of 15 herrons (5 Abassa / Sharkiya, 5 Altal Alkabeer / Sharkiya and 5 Lake Manzalla / Damietta) were pooled into three samples, each representing a single geographical locality (Table 2). Further, a total of 10 colacal swabs (2 pools) were collected from wild ducks (5 Bahr Albaqar / Sharkiya and 5 Lake Manzalla / Damietta). A total of 15 poultry manure samples (3 pools of 100 gm each) were collected from some of recently fertilized earthen ponds within the vicinity of Sharkiya (Abassa), Dakhleya (Lake Manzalla), and Damietta (Shatta) (Table 2).

Sample processing:

Nile tilapias and Red Swamp crayfish were transferred alive to the wet lab of the Fish Diseases and Management Laboratory (FDML) using well aerated insulated Styrofoam boxes. Pufferfish and

oysters were collected alive from Abou Qir Bay then transferred to the FDML using ice filled standard insulated ice boxes. Pooled samples from mouth parts, crusts and thoracic legs of adult Red swamp crayfish were finely homogenized using sterile homogenizer. Pooled sample of juvenile nymphal stages of crayfish were minced together using sterile pair of scissors then further homogenized. Homogenates were further diluted using Hanks balanced salt solution (HBSS: Sigma Chemical Co, St. Louis, MO, USA) (4 HBSS / 1 homogenate). Hemolymph samples from adult crayfish were collected using sterile syringes then aliquoted into 1 ml microfuge tubes. Diluted homogenates / hemolymph were stored at -80 °C freezer till processed.

All fish samples were washed up using 70 % ethanol before being dissected (Kidneys were retrieved from fish after opening under complete aseptic condition using three line incision). Mucous / fins mixture, gills and kidneys of Nile tilapia were finely homogenized using sterile homogenizer. Only blood samples were collected from the caudal vessels of sharp toothed catfish that were recently predating on dead bird carcasses as reported by the fish ponds owners (Plate 3-B). Blood samples were quickly aliquoted into 2 ml microfuge tubes and stored at 80 °C freezer till processed. Pufferfish kidney samples were transferred into sterile falcon tubes then further finely homogenized before being stored at -80 °C. Whole oysters were washed up using 70 % ethanol then flesh from each individual gastropod was dissected using sterile pair of scissors and forceps. Flesh tissues were transferred to 1 ml microfuge tube then finely homogenized using sterile homogenizer and stored at 80 °C freezer till processed.

Ducks and herons were trapped using duck traps and clap nets. Cloacal swabs were collected using cotton swabs and subsequently stored in transport media at -80ºC. Transport media consisted of Hanks balanced salt solution supplemented with 10 % glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulphate, 250 µg/ml gentamicin and 50 U/ml nystatin (All from, Sigma Chemical Co, St. Louis, MO, USA). In respect to poultry manure samples, random amounts (1gm from each pool) were stored in transport media at -80ºC till processed.

RNA isolation:

RNA was extracted from the collected samples using Trizol® LS Reagent (Invitrogen, Carlsbad, CA) or the QIAamp® Viral RNA Mini Kit (Qiagen Inc., Valencia, CA) following manufacturers' instructions with minor modifications. A 0.2 ml sample was homogenized by vortexing and subsequently lysed with 0.4 ml lysis/binding buffer. After binding to the column, DNase-I digestion and washing, the RNA was eluted in 50 µl nuclease-free double-distilled water. Initially, pools of 5 samples are tested (without significant loss of sensitivity).

RT-PCR:

Samples were amplified in a one-step RT-PCR in 25 µl final volume, containing 50 mM Tris. HCl pH 8.5, 50 mM NaCl, 7 mM $MgCl_2$, 2 mM DTT, 1 mM each dNTP, 0.4 µM each oligonucleotide, 2.5 U recombinant RNAsin, 10 U AMV reverse transcriptase, 2.5 U Ampli-Taq DNA polymerase (all enzymes from Invitrogen) and 5 µl RNA. Primers to detect any type A influenza viral genome located at M gene as well as the H5-specific primers were adopted from a previous protocol described by Saberfar *et al.* (2007) . The sequences of the designed primers were MF: 5' CTT CTA ACC GAG GTC GAA ACG 3' and MR: 5' AGG GCA TTT TGG ACA AAG CGT CTA 3') for M gene amplification. The used H5-specific primers were H5F: 5' ACG TAT GAC TAT TCA CAA TAC TCA G 3' and H5R: 5' AGA CCA GCT ACC ATG ATT GC 3'. Thermo-cycling was performed in a thermal cycler (Bio-Rad, Hercules, CA) using the following cycling conditions: 30 minute at 42ºC, 4 minutes at 95ºC once; and 1 minute at 95ºC, 1 minute at 45ºC, 3 minutes at 72ºC repeated 40 times.

3. Results

Freshwater fish and shellfish:

RT-PCR results for the hemolymph samples of the freshwater crayfish (*Procambrus clarkii*) collected from Abassa / Sharkiya have revealed that 3 out of 4 pools were positive for H5N1 virus while all hemolymph samples of Altal –Elkabeer crayfish were positive (4 out of 4 pools). A total of 2 out of 3 whole minced freshwater crayfish juveniles collected from Abassa / Sharkiya were positive for the virus while all samples collected from Altal –Elkabeer were negative for the virus. It is worthy to mention that all crust-legs-mouth parts samples collected from both locations were negative (Plate 2 B) (Table 1).

As a positive remark for the endemic existence of the virus in crayfish, we have found that all hemolymph samples collected from Port Said and Damietta earthen ponds were positive for the virus. Controversially, all Nile tilapia's mucous; fins, gills and kidney samples collected from Abassa / Altal Alkabeer Sharkiya were negative for the H5N1 virus. RT-PCR test has confirmed that all blood samples collected from the bottom feeder sharp toothed catfish at Lake Manzala / Damietta were positive for the virus (Plate 2 A) (Table 1).

Table (1) Fish and shellfish sampling details and Avian Influenza virus detection results

Sampled Species	Province	Sampling location	Aquatic habitat	Sampled stage	Average Weight	Sampled tissue	Number samples	RT-PCR results	
								# +	# -
Freshwater Crayfish	Sharkiya	Abassa	Earthen pond	Adult	50	Crust- legs - mouth parts	5 (1 pool)	0	1
				Adult	50	Hemolymph	20 (4 pools)	3	1
				Juvenile	20	Whole minced	15(3 pools)	2	1
		Altal Alkabeer	Earthen pond	Adult	70	Crust- legs - mouth parts	5 (1 pool)	0	1
				Adult	70	Hemolymph	20 (4 pools)	4	0
				Juvenile	30	Whole minced	15 (3pools)	0	3
	Port Said	Ashtoum Algamil	Earthen Pond	Adult	45	Hemolymph	40 (4 pools)	4	0
	Damietta	Lake Manzala	Earthen Pond	Adult	40	Hemolymph	40 (4 pools)	4	0
Nile tilapia	Sharkiya	Abassa	Earthen Pond	Fingerling	50	Mucous & fins	50 (5 pools)	0	5
						Gills	50 (5 pools)	0	5
						Kidney	100 (10 pools)	0	10
		Altal Alkabeer	Earthen Pond	Fingerling	50	Mucous & fins	50 (5 pools)	0	5
						Gills	50 (5 pools)	0	5
						Kidney	100 (10 pools)	0	10
Sharp toothed Catfish	Damietta	Lake Manzala	Earthen Pond	Adult	600	Blood	30 (3 pools)	3	0
Rabbitfish	Alexandria	Abou Qir Bay	Mediterranean Sea coastal water	Adult	1000	Kidney	25 (5 pools)	4	1
Mediterranean Cone Shell			Mediterranean Sea coastal water	Adult	50	Whole flesh	30 (3 pools)	3	0

Table (2) Birds sampling and Avian Influenza virus detection results

Sampled Species	Province	Sampling location	Aquatic habitat	Average Weight / gm	Sampled tissue	Number samples	RT-PCR testing results	
							# +	# -
Herons	Sharkiya	Abassa	Neighborhood of sampled Earthen ponds	1000	Cloacal swab	5 (1 pool)	1	0
		Altal Alkabeer		900	Cloacal swab	5 (1pool)	0	1
	Damietta	Lake Manzala		1050	Cloacal Swabs	5 (1pool)	0	1
Ducks	Sharkiya	Bahr Albaqar	Neighborhood of sampled Earthen ponds	1000	Cloacal swab	5 (1pool)	1	0
	Damietta	Lake Manzala	Neighborhood of sampled Earthen ponds	1050	Cloacal Swabs	5 (1pool)	1	0
Poultry	Dakhleya	Manzala	Earthen Ponds	100	Manure	5 (1pool)	1	0
	Damietta	Shatta		100		5(1pool)	1	0
	Sharkiya	Abassa		100		5 (1pools)	1	0

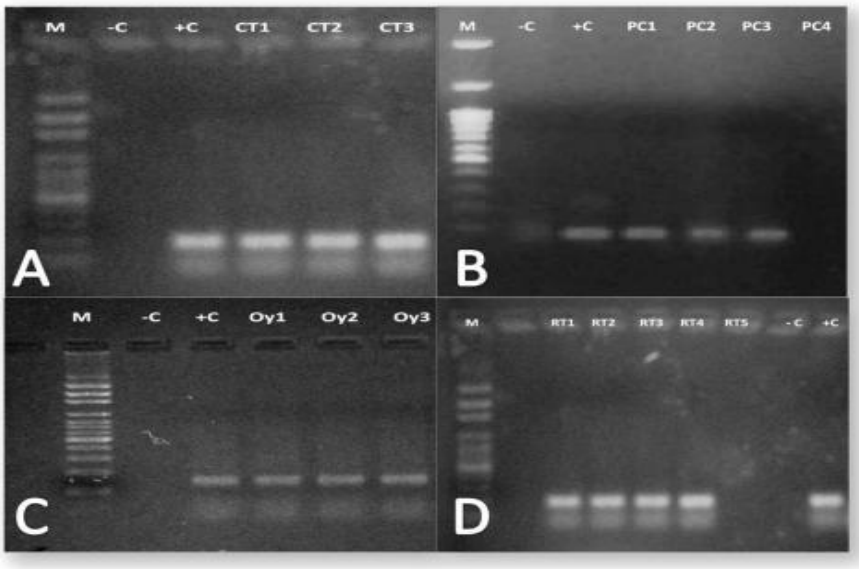

Plate (2) A. Agar gel electrophoresis showing specific bands for H5N1 positive catfish (CT) blood samples ; (2)B. Agar gel electrophoresis showing specific bands for H5N1 positive crayfish (PC) hemolymph samples; (2)C. Agar gel electrophoresis showing specific bands for H5N1 positive Mediterranean cone shell (OY) tissue samples; (2)D. Agar gel electrophoresis showing specific bands for H5N1 positive Pufferfish (RT) kidney samples.

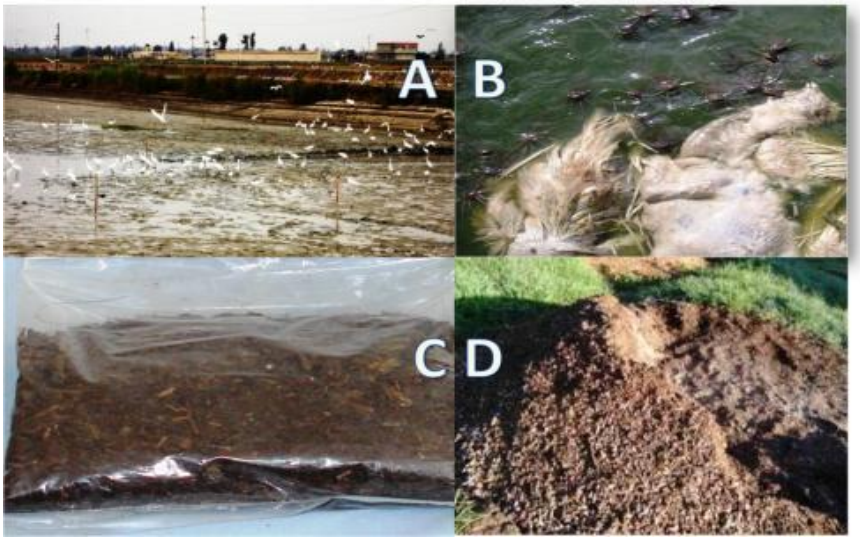

Plate (3)A. Herons dominating an aquaculture earthen pond; (3)B. Sharp toothed catfish predating on dead bird carcasses thrown at Manzala water body; (3)C. Poultry manure sample after collection ; (3) D. Poultry manure piles before earthen pond natural fertilization.

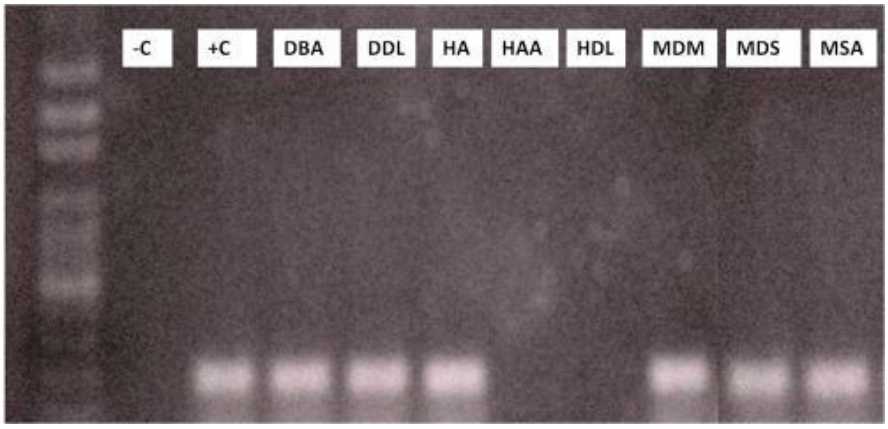

Plate (4) Agar gel electrophoresis showing the positive RT-PCR results of sampled aquatic birds: DBA: Ducks from Bahr Albaqar, Sharkiya; DDL: Ducks from Lake Manzala , Damietta ; HA : Herons from Abassa , Sharkiya; HAA: Herons from Altal Alkabeer, Sharkiya; HDL: Herons from Lake Manzala, Damietta ; MDM: Poultry manure from Manzala, Damietta; MDS: Poultry manure from Shatta, Damietta; MSA: Poultry manure from Abassa, Sharkiya

Marine fish and shellfish:

RT-PCR testing of the invasive Pufferfish (*Lagocephalus sceleratus*) collected from Abou Qir Bay , Alexandria has revealed that 4 out of 5 pools were positive for H5N1 virus (Plate 2 D) (Table 1) while whole flesh samples of the gastropod Mediterranean Cone Shell (*Conus mediterraneus*) were all positive (3 out of 3 pools) (Plate 2 C) (Table 1).

Poultry and manure samples:

RT-PCR tests indicated that cloacal swabs sampled from herons captured at Abassa / Sharkiya were positive while that of Altal Alkabeer herons were negative for H5N1 virus. On the contrary, cloacal swabs from Damietta's herons were negative for the virus. Remarkably, Ducks' cloacal swabs from Bahr Albaqar / Sharkiya as well as Lake Manzala / Damietta were all positive for the virus. Ultimately, all poultry manure samples collected from recently fertilized earthen ponds at Manzala/ Dakhleya, Shatta/ Damietta and Abassa / Sharkiya were all positive for H5N1 virus (Plate 4, Table 2).

It should be noted that all tested samples were subject RT-PCR testing for type A influenza virus using M-gene primers then positive samples were

further subjected to RT-PCR testing for H5N1 virus using H5 gene primers.

4. Discussion

During the past few years, Egypt has been stormed with numbers of influenza virus epidemics among human, poultry and swine populations. The peak of fatalities among human populations at different Egyptian provinces was mainly attributed to acute cases of swine flu (H1N1). On the other side, countable number of human mortalities scattered through vast geographical locations were mainly related to the vulnerable household rearing of different avian influenza (H5N1) possibly infected poultry populations (Aly *et al.,* 2008). However, the countable number of human cases was not reflecting the real crisis among the Egyptian poultry industry.

In 2006, the Egyptian government screening tools have discovered that a relatively large proportion of the poultry farms as well as backyard birds were positive for the virus (Aly *et al.,* 2008). This shocking news have triggered a very swift reaction from the veterinary authorities to stop the swift spread of the virus from state to another fearing the possible transmission to human populations Egypt's wide. By the end of 2006, millions of birds including farm, household and wild populations were subjected to total condemnation in a trial to eradicate the disease. Unfortunately, all control trials have faced absolute failure with an ultimate result that Office of International Epizootics (OIE) has listed Egypt among the avian influenza (H5N1) endemic states which became an existing fact till the moment (Aly *et al.,* 2008).

Throughout the past few years following the declaration of Egypt as an H5N1 endemic state, many researchers have been deeply investigating the real causes behind such national crisis. These investigations have involved numerous possible biological, environmental and administrative confounded artifacts that tragically ended with such national disaster. Researchers have followed several diversified assumptions which included but not limited to: application of faulty imported vaccine, un-tightened biosecurity procedures, improper eradication regimes, rearing of backyard poultry populations, wild bird migration flyways crossing north and south of Egypt, unhygienic disposal of dead birds' carcasses into the water bodies, habitual predation of carnivorous fishes on infected dead bird carcasses, predation of piscivorous birds on fish and shellfishes mechanically carrying the virus on/in their body surfaces/ tissues , and misusage of inefficiently treated poultry manure as aquaculture ponds fertilizers.

As an initial pilot study for our current 200 K Cairo university funded project titled "The roles played by shrimp, freshwater crayfish (*Procambrus clarkii*) and fish in the transmission and spread of Avian Influenza H5N1 at the Egyptian Territory "we have investigated the possible existence of the H5N1 influenza virus on/in surfaces, biological fluids and tissues of several fish and shell fish species from diverse geographical location scattered through Egyptian Nile delta. To be representative, we have collected samples from both open/closed aquatic habitats geographically located at the flyways of intercontinental bird migration as well as those neighboring poultry farms with previous history of endemic nature of H5N1 influenza virus.

Interestingly, all Nile tilapia (*O. niloticus*) mucus, fins, gills and kidney samples were free form the H5N1 viral particles which could attributed to the fact that cichlid family of fishes are surface feeders which means they selectively pick up their foods directly from the water surface. The earthen pond aquaculture facilities at Abassa / Altal Alkabeer, Sharkiya are open type facilities which utilize both artificial (floating or semi-floating pellets) and natural (surface water phytoplankton) foods which are the preferred type of food for Nile tilapias (Ita, 1980; World Bank, 1997; Popma and Masser, 1999). Thus, the sedimentation of H5N1 possibly infected piscivorous bird' droppings into the bottom of the aquaculture pond will deprive surface feeder fish like tilapia from apprehending such infected particulate matter. As a result of such gifted behavior, tilapias might not carry on the viral particles in / on their body tissues. Being omnivorous non predator fish (Ita , 1980; illay, 1990) , tilapias are hypothetically unable to attack dead bird carcasses while floating in pond water surface, hence, their possibility of carrying viral particles are relatively nil. All above mentioned assumptions might explain how Nile tilapia samples from two diverse geographical locations are negative for the virus.

Red swamp crayfish (*Procambrus calrkii*) is a bottom filter feeding shellfish which sweeps the pond's bottom predating on several benthic organisms (Momot *et al.,* 1978; Scott and Thune, 1986; Darrigren, 2002). Thus, crayfish can engulf bottom settled benthic organism which could be parts of the settled down poultry droppings infected with H5N1 virus or any other pathogens. The well documented fact entailing the accidental presence of some pathogenic viruses (e.g. parvo virus) in the hemolymph of crayfish (Edgerton *et al.,* 1997) could explain how H5N1 influenza viral particles were detected in the hemolymph of the majority of Red swamp crayfish collected from the earthen ponds or even open water bodies at three different provinces

(Sharkiya, Damietta and Port Said). The presence of the three provinces at the scope of major migratory bird flyways, crowd of poultry farms as well as the faulty usage of poultry manure as fish pond fertilizers might explain the possible existence of H5N1 virus in both water and pond's bottom (Melville and Shortridge, 2006; Bennum, 2006; Brown *et al.,* 2007) for enough period that could approach several weeks (up to 190 days in wild viral strains) (Brown *et al.,* 2007) before being uptake by the filter feeder crayfish.

The erratic dumping of dead poultry carcasses into the water bodies is an environmental catastrophe that might represents a potential source for interspecies infectious diseases' transmission. Further, the predator feeding behavior of sharp toothed catfish could allow them to attack the dumped dead carcasses which were originally derived from an H5N1 infected neighboring poultry farm around Lake Manzala. After having such possibly infected meal, the virus could circulate in catfish blood for reasonable time till completely cleared. The bottom feeding behavior of catfish might add another predisposing factor for contracting H5N1 viruses from the precipitated poultry manure coming from either swimming migratory aquatic birds or poultry manure pond' fertilizers.

The biodiversity of the East Mediterranean has been considerably altered since the opening of the Suez Canal in 1869. The pufferfish, *Lagocephalus sceleratus* (Bilecenoglu *et al.,* 2006) is an alien fish species that has invaded the Mediterranean seas causing violent ecological alterations after escaping their native habitat (Indian Ocean) due to diverse ecological, food zone and climatic changes (Halim and Rizkalla, 2011). Pufferfish are predominately feeding on dinoflagellates existing in the profound Mediterranean Sea 'depths (80 m), thus they are usual unintentional components of the fishing harvest by trawling machines at such areas of the Mediterranean (Golani and Levy, 2005; Aydin, 2011).

Interestingly, our RT-PCR screening assay has confirmed the presence of H5N1 viral particles in all examined kidney tissue samples of Pufferfish caught from Abou Qir Bay, Alexandria. The presence of H5N1 viral particles in kidney tissues could be derived from the feeding of pufferfish on possibly infected dinoflagellates followed by their digestion and sequential circulation in blood. Once viral particles got arrived to pufferfish blood, they could be lodged in their circulating phagocytes with an ultimate settling into the gut associated lymphoid tissues (GALT) and anterior kidney. This pathopysiological mechanism were imitated from similar viral infections belonging to the same influenza virus family (Orthomyxoviridae) infecting fishes such as infectious salmon anemia (ISA) (Kibenge *et al.,* 2006). However, other non orthomyxoviridae viruses (infectious hematopoietic necrosis) were following the same sequential pathogenesis in different fish species (Kim *et al.,* 1999). Some viral species such as white spot syndrome virus (WSSV), Noda virus and other DNA viruses have been reported to infect dinoflagellates in marine environment (Nagasaki *et al.,* 2006; Tomaru *et al.,* 2009; Soumya *et al.,* 2012). Similarly, H5N1 virus could be incidentally slotted into dinoflagellates inhabiting the profound zones of Abou Qir Bay where they might be fed by the existing pufferfish allowing the viral particles to be settled in the fish's GALT and anterior kidney hematopoietic tissues .

Concurrently, the H5N1 viral particles were also detected in all soft tissues of the filter feeder cone shell (*Conus Mediterraneus*) collected from Abou Qir Bay, Alexandria. The H5N1 viral particles were possibly reached the soft tissues of the cone shell either by incidental lodging via filter feeding on the settled marine aquatic birds' droppings or by feeding on infected oligochaete worms. Our assumption was inferred from the fact that oligochaete worms were reported to be vulnerable source of transmission to many filter feeder shellfish specific viruses such as WSSV and Noda virus (Vijayan *et al.,* 2005; Katsanevakis *et al.,* 2008; Sudhakaran *et al.,* 2008; Jones, 2012).

Wild birds are considered to be the natural reservoirs of avian influenza virus (AIV) (Webster *et al.,* 1992) and the Anatidae (in particular ducks), many of which are long distance migrants, generally have a higher incidence of infection than other birds (Webster *et al.,* 1992). In early 2006, the expansion of H5N1 outbreak range has continued into more central parts of Europe and the Middle East, together with Africa and India (FAO, 2005). The proximity of these outbreaks to water bodies has led wild water birds to be blamed for virus introduction, e.g. the OIE report on the deaths of poultry (ducks and geese) in Kazakhstan stated that the birds had contracted the virus through contact with wildfowl on open reservoirs (FAO, 2005). Duck flocks are also frequently led to nearby ponds and lakes during the day, and taken back to the homestead at night where they mix with the remainder of the backyard poultry flock (FAO, 2005). There is thus a degree of association between avian influenza outbreaks and wetlands in many parts of south-east Asia and this has led to suspicions (in some circles claimed certainty) that wild water birds have been responsible for the spread of the virus.

Coinciding with the above reported data, all pooled Cloacal swabs collected from number of wild ducks caught at the nearby of fish ponds throughout

two different provinces (Sharkiya and Damietta) were positive for H5N1virus. However, 50 % of Cloacal swabs collected from native wild herons caught at the nearby of fish ponds within two different sampling locations at Sharkiya province (Abassa and Altal Alkabeer) were positive for the virus. From different biological perspective, the predatory feeding behavior of migratory aquatic birds which naturally feed on aquatic invertebrates such as shrimps , crayfish and snails might set a reasonable hypothesis for their significant role in the epidemiological cycle of H5N1 virus in Egypt and worldwide.

The positive RT-PCR results of the poultry manure samples collected from earthen aquaculture ponds at three different Egyptian provinces (Sharkiya, Dakhleya and Damietta) prior to their usage a pond fertilizer, is highly suggest for a very critical role in spread and transmission of H5N1 virus through integrated aquaculture systems adopted by many Egyptian aquaculture investors. These results coincided with many worldwide published reports confirming the possible role of poultry manure and integrated aquaculture in the spread and outbreaks of H5N1 virus (Bennum, 2006; Brown, 2006; Melville and Shortridge, 2006).

5. Conclusion

In conclusion, the majority of the retrieved data in our two years study has exposed the critical importance of many aquatic species in creating an intermediary link for transmission, processing and spread of influenza viruses to and from vulnerable aquatic and poultry populations (Kwon *et al.*, 2005). Further, it is very imperative to conclude that erratic dumping of dead bird carcasses into water bodies as well as faulty usage of inefficiently treated poultry manure in organic fertilization of fish ponds would result in catastrophic eruption and evolution of new influenza viral hybrids with an ultimate disaster of state wide pandemic. Ultimately, extensive pathogenicity studies are essential to investigate the assumed probability of the H5N1 virus to adapt and replicate in aquatic animal modules.

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