

MOLECULAR DETECTION OF AVIAN INFLUENZA (H5N1) IN SOME EGYPTIAN CRUSTACEANS

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ABSTRACT

To check the liability of some marine and freshwater crustaceans to contract infection with Avian Influenza (H5N1) Randomly 268 different shellfish samples including 28 *P. clarckii* were collected from Abbassa, Sharkeya city and 240 marine samples (100 *P. japonicus*, 100 *P. semisulcatus*, 20 *C. sapidus* and 20 *P. pelagicus*) were collected from Suez-Gulf, Suez city and Murrah Lake, Ismailia city. Most of clinically examined Samples were apparently healthy. All marine shellfish samples (Shrimps and Crabs) were negative for H5N1 virus upon using RT-PCR utilizing the highly specific oligonucleotide primers where the 189 bp bands were not detected at any of the tested samples, while only 4/28 Red swamp crayfish were positive. Molecular screening using RT-PCR revealed no infection in all marine examined samples and remarkably showed that 14.4% of freshwater crayfish were positive for H5N1 virus.

Keywords:

H5N1- Red swamp crayfish - Avian influenza - Abbassa, Sharkeya - freshwater).

INTRODUCTION

Despite the fact that, the initial attempts of shrimp farming in Egypt goes back to about 10 years back, the productivity per unit area was never satisfactory due to variety of reasons. The farmed species is considered among those reasons. For years, green tiger shrimp, *Penaeus semisulcatus* has been tried but with modest productivity. The request to import whiteleg shrimp, *Litopenaeus vannamei* was rejected by fishery authority according to fishery law. Instead, the Indian white prawn was tried starting several years ago with better outcomes (**Sadek et al., 2002**). The crab yield, during the period from 2000 to 2015, increased from 19% to 42% of the total lagoon production. Moreover, the results show that, the crab catch in Egyptian fisheries is composed mainly of two crab species *Portunus pelagicus* (L.) and *C. sapidus* (Rath.). The latter is considered the most dominant in the crab yield with 85% of the total crab production, while *C. sapidus* only 15% (**Abdel Razeq et al., 2016**). Both crab

species had migrated to the Egyptian water. Thus, while *P. pelagicus* (L.) has an Indo-pacific origin and had migrated to the Mediterranean Sea through the Suez-canal, *C. sapidus* (Rath.) is native to western Atlantic coasts and widely recorded in various Mediterranean regions (**Lucrezia et al., 2015**). The red swamp crayfish, *Procambarus clarkii* has greatly affected rivers and lakes worldwide (**Nalepa and Schloesser, 1993; D'Itri, 1997**). *Procambarus clarkii*, also known as red swamp crayfish or Louisiana crawfish, is native to northeastern Mexico and the southern USA; it has been introduced in all continents except Antarctica and Oceania, being now considered the most cosmopolitan freshwater crayfish species in the world (**Gherardi, 2006; Chucholl, 2011**). In a general way, this species shows good tolerance to a wide range of environmental conditions, elevated adaptive capacity, high growth rate and flexible feeding strategy (**Alcorlo et al., 2004; Gherardi, 2006**), features that favor its establishment in new available habitats. Avian Influenza is a notifiable pandemic viral disease of poultry and wild birds (migratory and aquatic) caused by an orthomyxovirus Type A. the virus is not adaptable to fish or shellfish cells. However, it can be mechanically lodged on fish mucous, shellfish cuticle or in the intestinal tract or transiently passed to vital immunogenic organs like kidneys of fish or hemolymph of shellfishes (**Eissa et al. 2012**). The virus is phylogenetically related to ISA virus of Salmonids and TiLV of Cichlids. Globally, influenza virus has spread from country to country and from continent to another overseas through migratory birds flying pathways (from north to south and vice versa), commercial live poultry export and import and poultry smuggling between different neighbor countries through borders (**Furuse et al., 2010; Eissa and Hosni, 2015**). Influenza viruses were reported to spread from poultry to aquatic animals and vice versa. Marine animals (Whales, Seals, Otters) were reported to have H1, H3, H4, H7 and H13 influenza strains due to predation on infected aquatic birds (Sea gulls, ducks and penguins) (and mechanically or transiently infected aquatic animals (fish, shellfish, bivalves, snails) (**Scholtissek and Naylor, 1988; Feare and Yasué, (2006); Eissa and Hosni, 2015**). H1-2, H3, H4-7, H9-13, H15, H16 Influenza virus strains were reported to spread in wild aquatic environments through predation of fishes on filter feeder animals such as shellfishes (shrimps, lobsters and crayfish), bivalves and snails which might contain the virus for up to 21 days (**Eissa et al., 2012; Eissa and Hosni, 2015**). Such viral strains can also spread through predation of piscivorous birds on shellfishes (shrimps, lobsters and crayfish), bivalves and snails which might contain the virus for up to 21 days followed by virus processing in their gut and consequently releasing it

more virulent to poultry populations. They might also be capable of passing the virus with their droppings to settle down into the mucus of the cohabitating fishes which will be further eaten by another aquatic bird giving the virus dynamics an endless epidemiological cycle (Eissa *et al.*, 2012; Eissa and Hosni, 2015). Ingestion of droppings of infected aquatic migratory birds is a vulnerable route of spread of these viruses. In fish and shrimp culture, the use of inefficiently treated infected poultry manure in fertilization of earthen pond based facilities can result in spread of certain viral strains e.g. H1-12, H14, H15 within pond water where virus can stay viable in mud and manure for up to 21 days (Eissa *et al.*, 2012). Virus can be lodged on fish mucus while swimming in poultry manure fertilized earthen ponds and stays for 3 days in mucus till a piscivorous bird (ducks and seagulls) eat it then recirculate the virus after processing in their gut (Eissa and Hosni, 2015). Further, the filter-feeder shellfishes e.g. shrimp /crayfish can siphon the virus from the pond bottom where virus can be lodged within haemocytes at their haemolymph and / or in the intestine till being cleared after several days (Eissa *et al.*, 2012). Fishes and shellfishes are not yet known as specific host for the virus. Thus, they are considered as mechanical / transient biological carrier of the virus. Once carnivorous fish feed on infected dead bird carcasses, they virus find its way through the digestive tract either to blood and from blood to kidneys (hematopoietic organ) or from gut to be shed with feces into the water. In both cases virus can stay viable for up to 21 days without replication (Eissa *et al.*, 2012). Fish can feed on a filter feed animal (shellfishes, bivalves and snails) who were capable of engulfing the virus through their filter feeding behavior with consequent capture of the virus by their phagocytic haemocytes or by static effect of the hemolymph (Eissa *et al.*, 2012). Fish will be capable of digesting infected carrier shellfish releasing the virus into the gut which will find its way either to kidney after passing with blood or to the water with fish droppings (Eissa and Hosni, 2015). In all cases no signs of influenza have been reported in both fish and shellfishes.

MATERIAL AND METHODS

Sampling locations:

Marine shellfish samples (Shrimp and crabs) were collected from Attaka fishing port, Suez bay at Suez city and Murrah Lake at Ismailia city, Egypt while freshwater crayfish samples were collected from an earthen pond based facilities at Abassa, Sharkiya.



Fig. (1): Attaka fishing port on Suez bay from where the Samples were randomly collected.



Fig. (2): Murrah Lake at Ismailia city from where the Samples were randomly collected.



Sampling:

A. Shrimp.

A, total number of 200 natural adult penalized marine shrimp (50 *Penaeus japonicas* and 50 *Penaeus semisulcatus*) were collected from Attaka fishing port, Suez bay at Suez city and the same set of samples were equally collected from Murrah Lake at Ismailia City, Egypt.

B. Crab.

A total number of 20 adult Blue Crab (*Portugonus pelagicus*) were collected from Attaka fishing port, Suez bay at Suez city and 20 adult Chesapeake Blue Crab (*Canallectus sapidus*) were collected from Murrah Lake at Ismailia City, Egypt.

C. Crayfish.

A total number of 28 adult Red swamp crayfish (*Procambrus clarckii*) were collected from earthen pond based aquaculture facilities at Abassa, Sharkiya governorate, Egypt.

Methods:

Clinical examination of collected shellfishes:

Clinical examination was adopted on shrimp, crab and crayfish samples that showed possible disease signs as well as those apparently healthy. Shellfish exoskeleton was cleaned with cotton soaked in 70% ethyl alcohol. Clinical examination of the collected shellfish was done by naked eye for any possible body abnormalities using the method adopted from **Lightner and Redman, (1998)**. To apply PM examination on visually inspected shellfish samples, cleaning the surface of the cuticle by cotton soaked in 70 % ethyl alcohol then separation of carapace from underlying connective tissue using sterile scissors and forceps were done in clean space or under safety cabinet. Cutting out the Carapace sagittal to expose hepatopancreas for color/consistency and gills, foregut, midgut, hind gut, musculature, pereopods, pleopods, uropod, telson, gonads and genital organs for any deviation in size, color and consistency (**Lightner and Redman, 1998**).

Sample Processing:

The collected live samples were stored in an insulated ice-box till transferred to the Fish Diseases and Management Laboratory (FDML), Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Cairo University for further processing. Samples were collected during winter and onset of spring and summer seasons of 2016/ 2017 then processed for PCR according to protocols described by **Lightner and Redman, (1998)** for marine shrimp /crab samples and **Eissa et al., (2012)** for Red swamp crayfish. All collected shellfishes were cleaned, washed three times in sterile distilled water and dried thoroughly with sterile towels before dissection and collection of exoskeleton and internal organs. The carapace of the examined Shrimp /crab/ crayfish was split sagittally using a surgical scalpel and scissors. The selected tissues were finely homogenized using sterile

homogenizer till consistent homogenates were obtained. Homogenates were further diluted using Hanks balanced salt solution (HBSS: Sigma Chemical Co, St. Louis, MO, USA) (4 HBSS / 1 homogenate). Haemolymph samples from adult Shrimp / crab / crayfish were collected using sterile syringes then aliquoted into 1 ml microfuge tubes. Diluted homogenates / hemolymph were stored at -80°C freezer till processed for RNA extraction and PCR detection of Avian Influenza (H5N1).

RNA Extraction:

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.

Amplification:

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 MgCl₂, 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 (SimpliAmp, Thermo Fisher Scientific, USA-Catalogue#A24811) using the following cycling conditions: 30 minute at 42°C, 4 minutes at 95°C once; and 1 minute at 95°C, 1 minute at 45°C, 3 minutes at 72°C repeated 40 times.

Gel Electrophoresis

In two separate 0.1 ml microfuge tubes, five microliters of PCR product in 5 µl of 1X TAE Buffer and 1 µl (6X bromophenol) loading dye and 0.5 µl of DNA Ladder in 9.5 µl of 1X TAE Buffer plus 1 µl loading dye were consistently mixed then 10 µl of from each tube were loaded into a 2% agarose gel with ethidium bromide and electrophoresed at 100 V for 30-'32 minutes.

The resultant bands were viewed using UV Trans-illuminator (Spectronics Corporation, NY, and USA- Catalogue #TVC-312R). Gel pictures were documented using a digital camera (RX100 V The premium 1.0 type sensor compact camera with superior AF performance, Saint Diego, CA,USA).

RESULTS

Clinical Examination:

Shrimp.

Clinically, most of the examined shrimp (*P. semisulcatus* or *P. Japonicus*) from both Suez Gulf and Murrah Lake were apparently healthy.

Crabs.

All examined crabs collected from both Suez Gulf and Murrah Lake were apparently healthy with no record of lesions or parasites either externally or internally.

Crayfish.

Almost all examined crayfish samples from Abassa, Sharkiya were apparently healthy.

Molecular Detection:

All marine shellfish samples (Shrimps and crabs) were negative for H5N1 virus upon using RT-PCR utilizing the highly specific oligonucleotide primers where the 189 bp bands were not detected at any of the tested samples.

Only 4 out of 28 Red swamp crayfish were positive for the H5N1 virus documented by detection of 189 bp band.

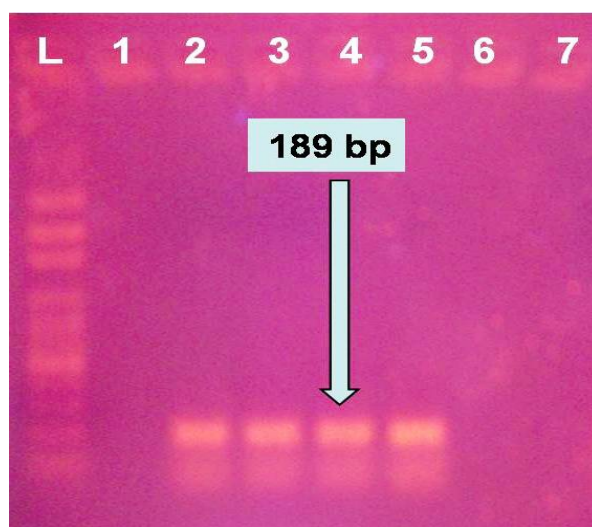


Fig. (4):The RT-PCR run showing the four H5N1 positive crayfish samples documented by appearance of 189 bp band on the ethidium bromide stained gel electrophoresis.

Total prevalence of AI (H5N1) infection among different examined species:

Molecular detection of the H5N1 influenza virus in total of 268 shellfish samples of different marine shrimp (*P.japonicas* and *P.semisulicatus*) and crab (*Port.pelagicus* and *C. sapidus*) species from both Suez gulf and Murrah Lake revealed negative results (0 out of 40) while the same PCR test revealed that 14.3 % (4 out of 28) of the tested freshwater crayfish (*Procambus clarkii*) from Abassa, Sharkiya were positive for the virus as shown in (Table 1) and Fig. (4). remarkably, all tested pooled tissue samples were positive for the virus with exception of crayfish flesh.

Table (1): Total prevalence of AI (H5N1) infection among different examined species.

Geographical location	Type of shellfish	Perevelence AI (H5N1)	Positive results
Suez gulf	<i>P. japonicus</i>	0%	0 / 50
	<i>P. semisulicatus</i>	0%	0 / 50
	<i>Port. pelagicus</i>	0%	0 / 20
Murrah Lake	<i>P. japonicus</i>	0%	0 / 50
	<i>P. semisulicatus</i>	0%	0 / 50
	<i>C. sapidus</i>	0%	0 / 20
Abassa , Sharkiya	<i>Procambus clarkii</i>	14.3%	4 / 28

DISCUSSION

Despite the fact that Influenza viruses are not adaptable to fish or shellfish cells. However, they can be mechanically lodged on fish mucous, shellfish cuticle or in the intestinal tract or transiently passed to vital immunogenic organs like kidneys of fish or hemolymph of shellfishes being phagocytosed by tissue fixed phagocytes or circulating macrophages (Eissa et al., 2012). Interestingly, most of orthomyxoviruses affecting fishes are active at freshwater environment e.g. Tilapia lake virus (TiLv) which affect Tilapia species in freshwater lakes and earthen ponds during summer season. However, another Orthomyxovirus like ISA mainly affects Salmonid species at seawater stage while carrier stage is linked to freshwater (Eissa and Hosni, 2015). In the current study, H5N1 virus was not detected at any of the marine shrimp and crabs collected from either Suez Gulf or Murrah Lake, which contradict the aforementioned findings for other orthomyxoviruses affecting aquatic species like ISA and coincides with TiLV, an emerging orthomyxovirus of the freshwater Tilapias.

This, could be attributed to the high salinity levels at both Suez Gulf (35-39 ppt) (Abd El-baky, 2012) and Murrah Lake (41-44 ppt) (El-Serehy *et al.*, 2014) which has inactivating effect on the virus infectivity as Brown *et al.* (2009) emphasized that avian influenza viruses could be inactivated at salinity levels higher 25 ppt. Interestingly, Red swamp crayfish (*P. calrkii*) is a bottom filter feeding shellfish which sweeps the pond's bottom predated on several benthic organisms (Momot *et al.*, 1978; Scott and Thune, 1986). 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 and Owens, (1997) could explain how H5N1 influenza viral particles were detected in the hemolymph, exoskeleton and internal organs of the 14.3 % of the Red swamp crayfish collected from the earthen ponds at Abassa, Sharkiya. The presence of water bodies of Sharkiya province 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; Stallknecht and Brown, 2007) for enough period that could approach several weeks (up to 190 days in wild viral strains) (Stallknecht and Brown, 2007) before being uptake by the filter feeder crayfish. Our results were completely consistent with Eissa *et al.*, (2012) who reported the presence of H5N1 virus in hemolymph and crusts of Red swamp crayfish with the exception of their flesh which were totally negative for the virus. In conclusion, the current study sheds the light on the magnitude of Influenza virus (H5N1) among wild shrimp, crabs and crayfish populations within the Egyptian open waters. Specifically, we declared that Suez Gulf and Murrah Lake shrimps and crabs populations were refractory to avian influenza (H5N1) infection. Presence of positive freshwater crayfish samples for Influenza (H5N1) confirms the critical epidemiological importance of such invasive aquatic species in spread and establishment of emerging viruses across the Egyptian aquatic habitats as well as their critical significance as a bridge in the Influenza cycle among wild aquatic bird's populations. Ultimately, results confirm previous assumptions of inability of avian influenza virus (H5N1) to survive higher salinities exceeding 30 ppt.

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