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The Possible Role of *Litopenaeus vannamei* shrimp in the transmission of Avian Influenza virus H5N8 in Saudi Arabia

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Abstract: Avian influenza is a highly contagious disease and poses a threat to both human beings and animals. The global spread of the disease in bird populations represents a major problem. Aquatic habitats aids in avian influenza virus transmission by many diverse hosts. The aim of this study was to screen crustaceans, namely, *Litopenaeus vannamei* shrimps for avian influenza virus and investigate their possible role in the virus transmission. A total number of 1200 adult live *Litopenaeus vannamei* were collected from Jazan Region, South-West coast of Saudi Arabia. RNA isolation and RT-PCR Techniques were performed in collected haemolymph. Results of virus detection by RT-PCR were negative, indicating that *Litopenaeus vannamei* may not have a role in the transmission of avian influenza virus in the Kingdom. Based on the current study, shrimps should be investigated in different geographical areas to access its possible role in avian influenza virus epizootic and epidemic cases in the kingdom.
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Keywords: Avian influenza virus, H5N8, *Litopenaeus vannamei*, RT-PCR Techniques, KSA.

1. Introduction

Avian influenza disease is an acute infectious disease caused by avian influenza viruses (AIVs) in domestic and wild birds, causing large socioeconomic losses (de Graaf and Fouchier, 2014; Medina and García-Sastre, 2011). The virus poses a threat to human and animal health as some AIV subtypes undergo antigenic shifts and can break the species barrier leading to pandemics or epizootics (Klenk et al., 2011). Recently, human infections with various AIVs were reported in countries such as Australia, Bangladesh and China (Obuchi and Tashiro, 2010). Thus, risk assessment and monitoring the circulation and gene flow of AIVs in their natural reservoir is crucial to understand the subtypes implication in epidemiology.

Wild aquatic birds represent important natural reservoirs in which AIV is transmitted either by direct contact or via the ingestion of water contaminated by other infected hosts as well as a cross-species transmission (Chan et al., 2013). Water compartments play a crucial role in AIV dynamics and the long persistence of the virus in the environment (Vittecoq et al., 2017). Although this second route of transmission is of utmost importance in AIV persistence and transmission in the environment, yet it has received far less attention than direct bird-to-bird contamination.

Aquatic habitats utilized by wild and domestic birds serves as a bridge for avian influenza virus transmission among many diverse hosts (Stallknecht et al., 2010). Avian influenza (H5N1) was detected in haemolymph of red swamp cray fish in Egyptian aquatic habitats (Eissa, 2012). In addition, an experimental model proved the accumulation of a low pathogenic AIV in zebra mussels *Dreissena polymorpha* (Stumpf et al., 2010) this highlight the possible role of aquatic organisms as a potential source of natural avian influenza infection. Thus, risk assessment that begins by monitoring the circulation and gene flow of AIV in their different hosts is crucial (WHO 2011).

In the Kingdom of Saudi Arabia (KSA), an outbreak of AIVs in commercial ostrich flocks were first reported during 2007. H5N1 strain was identified in the diseased birds by hemagglutination assay

followed by hemagglutination inhibition and real-time reverse transcription RT-PCR (rtRT-PCR) techniques (Monne et al., 2008). A second outbreak of avian influenza virus (H5N8) was reported in seven Saudi Arabia regions namely, Riyadh, Eastern Province, Al-Qasim, Makkah, Al-Madinah, Asir Region, and Jazan (Figure 1), with severe economic losses (Al-Ghadeer et al., 2018), this prove the rampant spread of AIVsin Saudi Arabia.

PCR-based diagnostic methods have been widely used as effective laboratory techniques for detection and genotyping of AIVs during outbreaks including conventional PCR, reverse transcription PCR (RT-PCR), and real-time rtRT-PCR (Elizalde et al., 2014; Hoffmann et al., 2016; Tsukamoto et al., 2012, 2009). Moreover, recent researches reported a multiplex RT-PCR method that simultaneously detect and differentiate multiple AIV subtypes (Hu et al., 2012; Li et al., 2018; Zeng et al., 2014).

The current study aimed to investigate the possible role of shellfish in the outbreak occurrence of avian influenza (H5N8) in the Saudi Arabian natural aquatic habitat, this will be performed through RNA isolation, RT- PCR and sequencing.

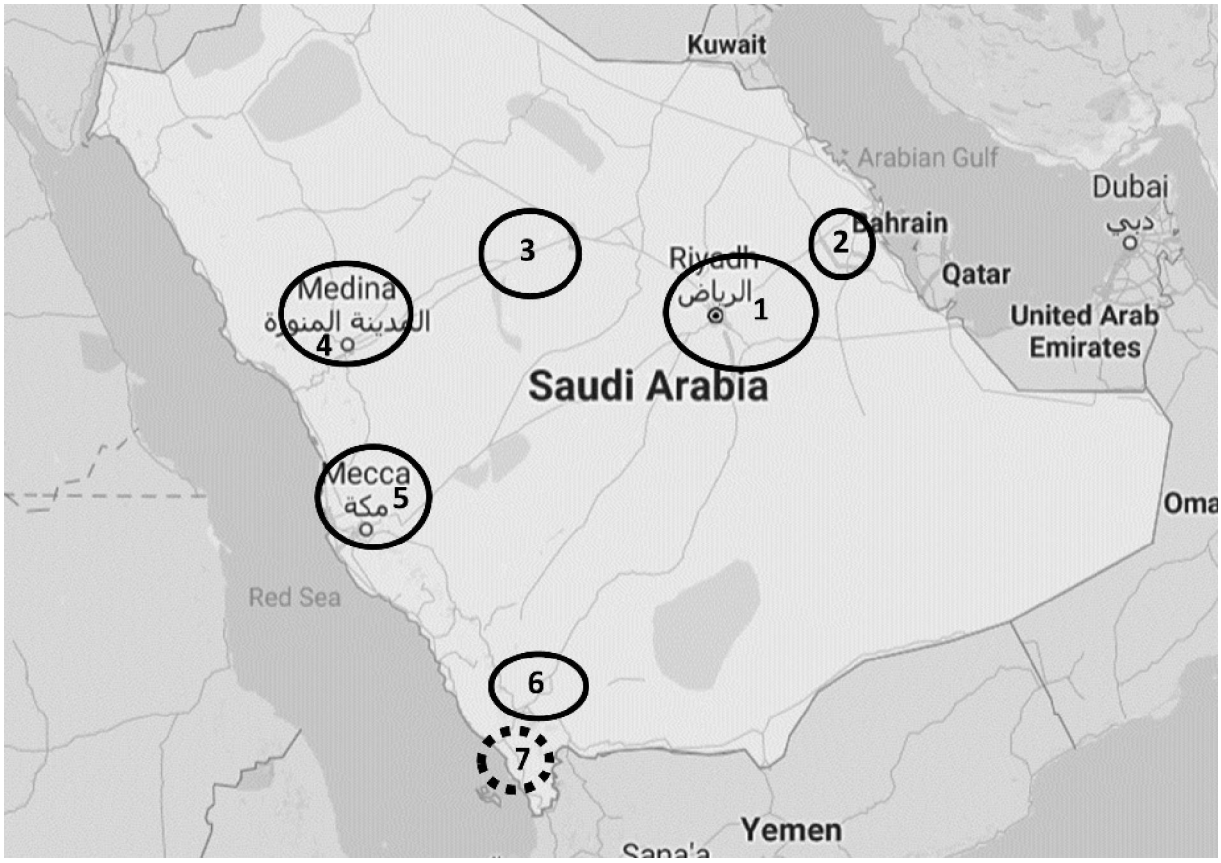

Figure 1. Geographic locations in Saudi Arabia in which outbreaks of H5N8 strain was documented in seven provinces: 1. Riyadh 2. Eastern Province 3. Al-Qasim 4. Makkah 5. Al-Madinah 6. Asir 7. Jizan; where the samples were collected (Al-Ghadeer et al., 2018)

2. Materials and Methods

2.1 Sample collection and geographic locations

A total number of 1200a dult *Litopenaeus vannamei* live shrimps with an average weight of 6–10 g each, were collected from Jazan coastal area, located in the Saudi Arabian South-West coastal region (Figure1), by the aid of local fishermen. Samples were collected in between September- November, 2018 in which water temperature was 25-28°C in average. Shrimps were transported alive to King Khalid University in Abha city for processing. Shrimps were acclimated in aerated artificial seawater aquaria (Salinity 22%, w/v) at 22°C, fed on commercial diet daily for two days before use.

2.2Haemolymph Collection and Processing

Shrimps were euthanized using chloral hydrate (Sigma, USA) prior to sample collection. The ventral sinus was flushed with 70% ethanol to sterilize the surface from which haemolymph were collected. From each shrimp, 1 ml of the haemolymph was drawn separately using a 5ml sterile syringe fitted with a 26-

gauge needle. The haemolymph was placed directly onto a sterile cryo-tube and used for RNA extraction.

2.3. RNA isolation and RT-PCR

Total RNA was extracted from shrimp hemolymph samples using the TRIzol ™ LS Reagent (Invitrogen, Thermo Fisher Scientific, USA) following the manufacturer's instructions. cDNA synthesis was performed using ImProm-II Uni12 Reverse Transcriptase System kit (Promega, USA) with 1µg of total RNA and 0.5µg of reverse primer (Table 1), which is complementary to the 12 conserved nucleotides at the 3´end of the vRNA which the eight negative-sense vRNAs (Figure 2), to synthesize the eight negative-sense viral RNA segments.(Hoffmann et al., 2001) The cDNA samples were then used in PCR detection of the target genes using forward and reverse primers (requested from eurofins genomics, Germany) complementary to the HA and NA specific regions (Table 1). PCR amplification was performed in a Bio-Rad thermal cycler and a thermal program consisted of an initial denaturation step at 94ºC for 4min followed by 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The thermal program ended with one cycle at 72 C for 7 min. PCR products were electrophoresed on ethidium bromide-stained agarose gel at 1% to detect the amplified PCR products of the target genomic sequences.

Table 1. Primer sequences for RT-PCR.

Primer	sequence		Product size (bp)
Uni12	Reverse	GGAACAAAGAUGA	
HA	Forward	TATTCGTCTCAGGGAGCAAAAGCAGGGG	1778 ± 29
	Reverse	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	
NA	Forward	TATTGGTCTCAGGGAGCAAAAGCAGGAGT	1413 ± 29
	Reverse	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	

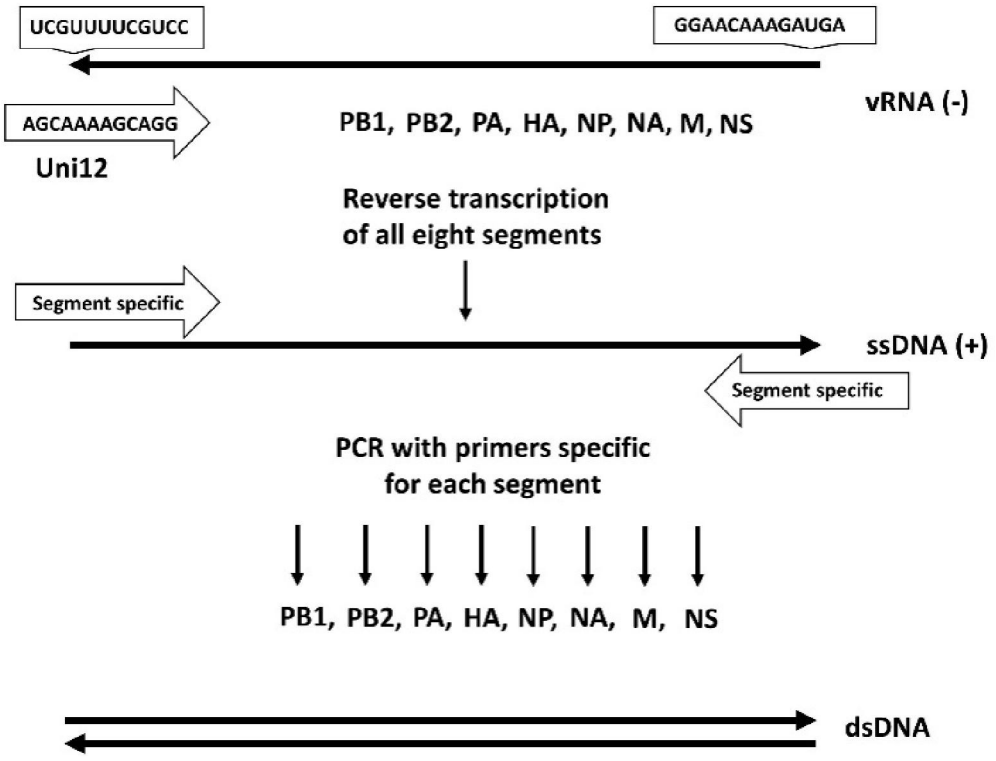

Figure 2. Schematic representation of the two steps RT-PCR using Uni12 primer for reverse transcription of the eight negative-sense viral RNA segments followed by PCR amplification of HA and NA specific genomic segments. Adapted from Hoffmann *et al.*, 2001.(Hoffmann et al., 2001)

3. Results and Discussion

Having collected live shrimp for the investigation of the role of these crustaceans in the transmission of AIV, precautions were taken to reduce any possible degradation of viral RNAs in any infected shrimp. Of all shrimps tested in this investigation, no positive result was observed following cDNA synthesis and PCR amplification.

Numerous studies investigated the possible role of aquatic environment in transmission of avian influenza virus. The increasing reports regarding AIV rapid spread in several continents and different geographic location surged the need for a detailed study on the methods of its spread to conduct a proper surveillance and control of the disease. The current study investigates the possible role of aquatic crustaceans, namely *Litopenaeus vannamei*; a common reared shrimp found in the Red Sea, in the transmission and spread of AIV in Saudi Arabia.

Litopenaeus vannamei SPF (specific pathogen free) modified shrimp broad stock was introduced to KSA as a biosecurity strategy to the regional level to overcome several shrimp diseases and disorders caused by the previous reared the Indian prawn species *Penaeusindicus* (FAO 2018). This can possibly explain the negative results of RT-PCR to AIV (strain H5N8) in our study.

Jazan area is located in the South Western region of KSA that is considered a main bird migration route exists along the western coastal plain of Saudi Arabia to the Southwest Arabian Peninsula/East Africa area. Migratory birds include geese, ducks and cranes, in addition to hawks which may be encountered year round in mountainous areas and sea gulls near populated coastal areas(Monne et al., 2008). The migration extends during the months of March to May and September to November.

On 2017, Al-Azeeziah Market in Riyadh was closed due to the outbreak of H5N8 that caused mortality for a vast number of birds with high economic losses(Al-Ghadeer et al., 2018). The Veterinary Diagnostic Laboratory in Riyadh recorded 14 affected cases, detrimental rigorous actions were taken by the Ministry of Environment, Water and Agriculture, KSA to control the spread of infection, this was done through hygienic destruction and disposal of all suspected birds and prevented the transmission and selling of birds within a circumference of 10km. In addition, extensive field and scientific researches are needed to assess the risk and thus aid in its control.

Conclusion

In this study, a rapid and sensitive real-time PCR assay was performed, for the detection and quantitation of AIV in live shrimp, *Litopenaeus vannamei*, samples. The results in this study can help us understand the possible distribution and transmission pattern of AIV *in vivo*.

This research came as trial to identify the role of shellfish, shrimp, in the transmission of AIV in Saudi Arabia, investigation of more samples numbers, regions and other types of shellfish is needed to determine the possible sources for AIV epidemiology in KSA.

Conflicts of interest

There are no conflicts of interest

Ethical approval

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Authors contributions:

Experiments design: Mai Ibrahem, Hanan Ogaly. Performing the experiments and data analysis: Mai Ibrahem, Hanan Ogaly and Abdulrahim Hakami. Writing the manuscript: Mai Ibrahem, Hanan Ogaly, Abdulrahim Hakami, Attalah Elkott.

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