Short Communication

The First Study of West Nile Virus in Feral Pigeons (*Columba livia domestica*) Using Conventional Reverse Transcriptase PCR in Semnan and Khorasane-Razavi Provinces, Northeast of Iran

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Abstract

Background: West Nile Virus (WNV) is an arboviral infection continuing to be as major threat to human health as well as the livestock industry all around the world. Birds including pigeons are one of the potential reservoirs for WNV. This study aimed to detect the presence of WNV genome in feral pigeons circulating in Semnan and Khorasane-Razavi Provinces (Iran) including 10 urban and 10 suburban areas.

Methods: Totally, 150 samples (brain and kidney) were collected equally from feral pigeons and the presence of WNV genome was evaluated in these samples after RNA extraction.

Results: All the samples were negative for the presence of WNV-RNA in this investigation.

Conclusion: Although obtained result indicated no evidence of WNV genome in feral pigeons but complementary studies regarding serologic detection of WNV in vertebrate hosts as well as pigeons and identification of arthropod vectors seems necessary for comprehensive determination about infection status in these areas.

Keywords: Feral Pigeons; West Nile Virus; RT-PCR; Iran

Introduction

West Nile virus (WNV) is a mosquito-borne virus related to genus *Flavivirus* (Flaviviridae) with nearly worldwide distribution and spread by mosquitoes belonging to the genera *Aedes*, *Anopheles*, *Culex* and *Ochlerotatus* (1-3). Historically, WNV infection was first identified from human cases with encephalitis in Africa (1937) and its significance as a zoonotic virus was highlighted (1). West Nile Virus infection shows wide distribution characteristics throughout southwestern Asia, Russia, the Middle East, Europe, Australia, and Africa. The worldwide distribution of WNV has changed during the last decades by the synergism of predisposing factors such as modern transportation, global warming, and demographic changes (4). Previous studies have demonstrated that the WNV has been detected in at least 27 provinces of Iran in different hosts including horses, humans and birds (5).

In nature, WNV circulates in birds and mosquitoes feeding from birds as biologic vectors and these hosts are characterized as its natural hosts at which the virus amplifies in their body. For WNV, humans and horses are expressed as accidental dead-end hosts notifying its zoonotic potential more (6). West Nile Virus infection has been reported from different continents as well Asia. The Iranian Semnan and Khorasan-Razavi Provinces are situated in the central and northeastern geographic parts of Iran and these two provinces are well-known for their

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suitable conditions for migratory birds from other countries in cold seasons. Migratory birds are one of the major reservoirs of WNV for other avian (pigeons) species and for arthropod hosts and virus distribution occurs via different roots of transmission such as feeding conditions (7). Previous studies confirm the existance of WNV in pigeons and their possible role in spreading of WNV to mammalian hosts (8-10).

Cities and livestock farms are considered as two suitable places for pigeons to make colonies because of the existence of food and for nesting purposes. So, these birds are now well adapted to farms and cities. Recently, there has been some concern about zoonotic pathogens that can be transmitted via pigeons to humans and domesticated animals as well horses. Specially, when such bird species co-exist with humans and livestock, they are considered to be a high risk factor for transmitting avian-derived pathogens such as WNV because of the strong chance of contact with mammalian hosts (8). In Iran, some investigations have confirmed the seroprevalence of WNV infection in humans, equines and wild water birds (birds circulating in water like ducks) and genome (WNV RNA) detection in mosquitoes but WNV RNA-detection found in these studies are very limited (11, 12). However, despite the presence of WNV genome in mosquitoes and human cases with encephalitis from Iran (11, 13), there is no information about the existance of WNV in free-living pigeons in Iran.

Therefore, a cross-sectional (5 years) study was carried out to investigate the molecular prevalence of WNV in feral pigeons from Semnan and Khorasane-Razavi Provinces (Iran) to identify the probable role of these birds as reservoirs of WNV in this two regions.

Materials and Methods

Sample collection

In this cross-sectional study, the sample size was calculated using the following equation: n= 4 PQ/L2, where n represents the minimum sample size needed for the prevalence estimation, P: prevalence (assumed prevalence of WNV in pigeons of the screened areas was considered 50% because of unavailability of previous data regarding its prevalence), Q: 100–P, and L: allowable error or precision (considered 0.1 in the present study). Then, the minimum required sample size became 100 samples from birds.

Brain and kidney samples were taken based on sterile procedure from deeply injured, euthanized with ketamine (30mg/kg) or freshly dead wild pigeons which had been admitted to different vet clinics located in Semnan and Khorasene-Razavi Provinces during a 5-year period.

The study was performed on 150 pigeons from different regions of (including 70 samples from 10 urban and 80 samples from 10 suburb areas) Semnan and Khorasane-Razavi provinces (Fig. 1) in north-eastern Iran during for autumn 2014 to winter 2019. In the present study, the 70 samples from urban regions were collected from six cities located in Semnan Province including: Semnan (35.5537810°N, 53.3791795°E), Damghan (36.1747867°N, 54. 3542161°E), Shahroud (36.3917606°N, 54. 9941698°E), Biyarjomand (36.0971499°N, 55. 8126512°E), Mayamey (36.4227077°N, 55. 6451097°E), Forumad (36.5198894°N, 56. 7492357°E) and four cities located in Khorasane-Razavi Province including: Sabzevar (36. 2301945°N, 57.6391283°E), Davarzan (36. 3431047°N, 56.8838182°E), Sheshtemad (35. 94831686°N, 57.7599779°E) and Rudab (36. 0283218°N, 57.3040453°E). Also, the 80 samples from suburbs were collected from five regions located in Semnan Province including: Ebrahim Abad (36.4160772°N, 55.7220140°E), Abkhori (35.8064424°N, 53.8563982°E), Ala (35.5431662°N, 53.4924760°E), Delazian (35. 4978981°N, 53.4066454°E), Armian (36. 3630742°N, 55.4031629°E) and five regions located in Khorasane-Razavi Province including: Kahak (36.3584244°N, 56.7727503°E), Mazinan (36.3156334°N, 56.8212518°E), Sadkharve (36.3119113°N, 57.0695328°E),

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Chesham (36.1717463°N, 57.0416171°E) and Karrab (36.3553765°N, 57.5003219°E).

These birds were not vaccinated against any viral agents, previously. Generally, sampling was accidental and kidney and brain samples were collected from mentioned birds and stored at −70 °C in sterile falcon tubes until performing RNA extraction.

Viral RNA extraction and RT-PCR amplification

The experiment was carried out using conventional Reverse Transcriptase PCR (RT-PCR) technique. Total RNA was extracted from brain and kidney samples using RNX- Plus Solution (Sinaclon, Iran) as described by the manufacturer's instructions. Then, cDNA was synthesized from 500ng of total RNA using CycleScript Reverse Transcriptase kit (Bioneer, Korea). Detection of WNV was carried out by amplification of the 3-untranslated region (3-UTR) using previously described primer pair (14). For all RT-PCR reaction sets, a plasmid containing the 3-UTR region of WNV was used as positive control and distilled H2O as negative control. Each 25µL PCR reaction consisted of 2× concentration mastermix containing polymerase, dNTPs, MgCl2, reaction buffer, and stabilizers (Jena Bioscience, Germany) by adding 0.8µL of each primer (10µmol/L; forward primer, CAGACCACGCTA CGGCG; reverse primer, CTAGGGCCGCGT GGG) and 50ng of template cDNA quantified by Nanodrop® (Smart Nano, Canada). Reaction mixes were overlaid with mineral oil and subjected to one step of 94 °C for 3min and then 35 cycles of amplification in a thermocycler (BIOER XP Cycler, China). The cycling condition was as follows: denaturation at 94 °C for 3min, annealing at a temperature specific (achieved by gradient temperature conditions) to the primer pairs 55 °C for 40s, and extension at 72 °C for 60s. Then, a final extension step was followed at 72 °C for 5min for completion of the last PCR cycle. Polymerase chain reaction amplicons (8–10µL) were electrophoresed in 3% agarose (Sigma-Aldrich) gel, stained with ethidium bromide (Sigma-Aldrich), visualized and photographed under a UV illuminator (Nanolytik™, England). Midrange DNA ladder (100 bp, Jena Bioscience) was used for fragment size determination.

Results

The molecular experiment amplified the expected fragment of DNA (536bp) in the positive control but all the 150 kidney and brain samples tested for the presence of West Nile Virus RNA by using conventional RT-PCR were negative as shown in Fig. 2.

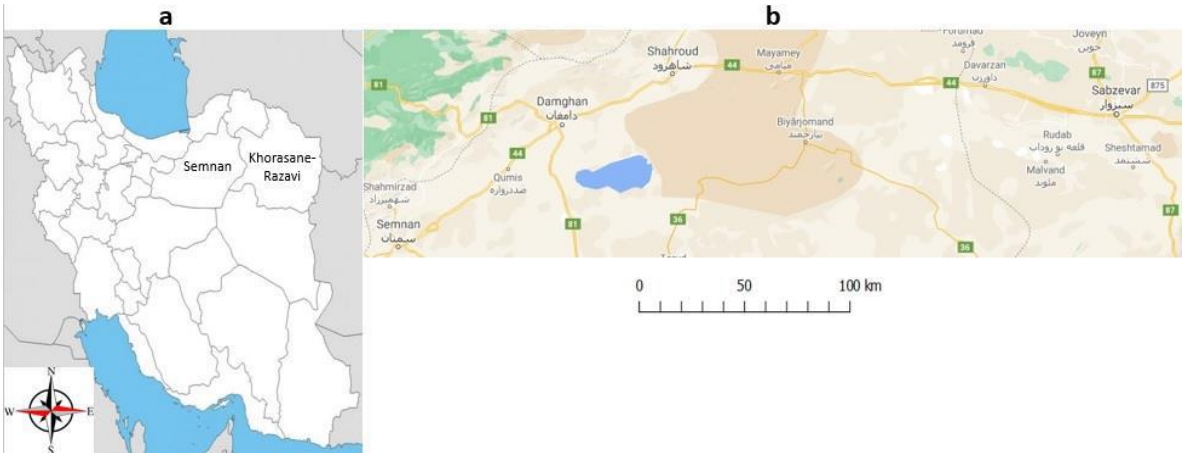

Fig. 1. Location of Semnan and Khorasane-Razavi Provinces in Iran (a). The magnified area of both provinces at which the sampling regions are distributed within the area (b)

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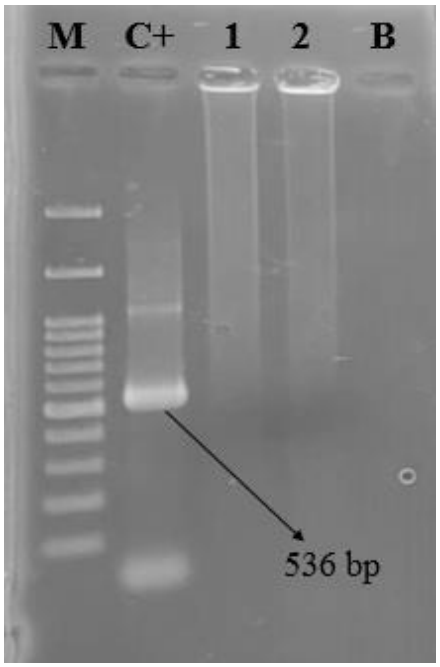

Fig. 2. Gel electrophoresis results of detected gene fragment regarding the West Nile Virus in the brain and kidney samples of screened pigeons. M: Marker (100bp), C+: Positive Control (3-UTR region of WNV weighting 536bp), B: Blank (Negative Control), 1-2: Samples showing no amplified fragments (negative samples)

Discussion

WNV genome was detected in none of the 150 screened pigeons in the present study. Previous publications have documented the role of migratory birds in the epidemiology of WNV. Also, field and experimental studies have shown that the pigeons have high WNV seroprevalence rates in endemic areas and can reproduce significant levels of viraemia (15-17). Some investigations have confirmed the presence of WNV genome (RNA) in pigeons in Asian countries such as Korea (8). However, to the best of our knowledge, no detailed study aimed at evaluation of the role of pigeons as reservoir for currently circulating Iranian WNV strains has been performed. According to the results regarding the absence of WNV genome in the pigeons of monitored areas in the present study, it is highly recommended to perform a serologic survey on these bird species to find out their probable previous exposure to WNV and their seropositivity. The absence of WNV in these areas and in vertebrate hosts as well pigeons, the very low virus loads in the samples and region's weather conditions in sampling seasons may provide unsuitable circumstances for the presence of WNV in screened areas.

West Nile virus is an emerging infectious pathogen for a variety of hosts including mammals, birds and even reptiles with a worldwide geographical distribution as well as Asian countries (18, 19). Migratory birds species play an important role in spreading WNV and specific species of birds are considered as critical reservoirs for this viral agent because the ornithophilic arthropods especially Culex spp. feeding on birds play important role in the natural cycle of WNV infections (23, 25-26). Then, birds circulating around humans and farm animals can introduce WNV to these vertebrate hosts via infecting the Culex mosquitoes and their bites, subsequently.

Previous investigations have confirmed the presence of WNV infection in Iran and neigh-

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boring countries via serologic and virological assays (11). The first report regarding the presence of WNV in Iran is documented by Naficy and saidi (22) by serological assays in human cases and higher rates of seroprevalence have been observed in Central and Southwestern Iran. Because of the presence of different climatic conditions and theoretically suitable environments for the establishment of WNV foci across Iran, different investigations have focused on the existence of WNV in arthropods species (11), humans and equine hosts (22-26), and migratory and water birds (19) representing a heterogeneous geographic distribution of WNV in different regions. However, despite the extensive distribution of feral pigeons as potential vector species, there is no information about the existence of WNV in this bird populations and monitoring of feral pigeons in Iran seems to be necessary as a factor involved in the cycle of this disease because previous studies have introduced pigeons as reservoirs of WNV (8).

In the present study, WNV genome was not detected in screened birds in Semnan and Khorasane-Razavi regions of Iran. However, the reasons of these negative results can depend on various impacting factors.

Conclusion

In conclusion, although our result indicated no evidence of WNV infection in feral pigeons but serological surveillance of these birds as potential vectors for WNV is highly recommended to fully understand the actual statues of this infection. For this reason, our investigations will continue for the detection of antibodies against WNV in vertebrate reservoirs including pigeons and determination of mosquitos' fauna regarding to this viral pathogen existing in these regions.

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