Original Article

Molecular Evaluation of the Novel Coronavirus Infection of Cockroaches and Flies Collected from Kamkar-Arabnia Hospital in Qom City, Central Iran: With Innovated Internal Control

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Abstract

Background: Due to the confirmation of the presence of the novel coronavirus in the feces and municipal sewerage system, and the feeding of domestic insects from fecal matter, as well as the ability of these insects to mechanically transmit microbes from the sewerage system. This study was aimed at molecular evaluation of the novel coronavirus infection isolated on cockroaches and flies collected from Kamkar-Arabnia Hospital in Qom City, Iran.

Methods: Totally, 18 samples; (12 samples cockroaches and 6 flies) from the external surface of cockroaches and houseflies as well as their digestive system were prepared. After designed and synthetized exogenous heterologous internal control, the RNA was extracted to investigate the contamination of these samples with the novel coronavirus. To detect the virus, the E and RdRp genes were identified.

Results: Investigation of coronavirus E gene using the multiplex one-step qPCR technique on the collected samples showed an amplification plot in CT= 35.70 related to the internal surfaces of cockroaches collected from the treatment and sick room of the hospital. Also, the design of internal control to ensure the accuracy of the extraction process was successful.

Conclusion: According to the findings of the present study regarding detecting the presence of the coronavirus infection in the digestive system of domestic insects such as American cockroaches and considering their ability to mechanically transmit viruses, it is recommended to control the domestic insects that are in close contact with humans in crowded places such as hospitals and health centers during the Covid-19 pandemic.

Keywords: Cockroaches; Flies; Hospital; Novel coronavirus infection; One-step qPCR; Iran

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a causative agent of a zoonotic disease that emerged in the late Dec 2019 in China and caused of the coronavirus disease19 (Covid-19) pandemic (1). This novel virus is classified as an RNA virus belonging to the coronaviridae family (2). This family of viruses is the

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causative agent of a wide range of viral diseases, from the common cold to more severe respiratory infections such as Middle East Respiratory Syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV). The coronaviruses have so far attracted a great deal of attention from international viral pathogenesis and pathophysiology (3). In fact, nowadays, the novel coronavirus, which causes Covid-19, is more lethal than the other coronaviruses and is a serious warning to all countries in the world (4). As of October 26, 2020, 08:43 GMT, more than 43 million people (43,376,552) worldwide have been infected with the virus, and more than 1.1 million deaths (1,159,534) have occurred, according to the World Health Organization (WHO) (5). The disease has adverse effects on public health and has affected other social and economic aspects of the population (6). The Covid-19 is a highly contagious disease, and each infected person can infect an average of at least 3 others (7). Human-to-human transmission (HHT) of the virus through respiratory droplets and contaminated objects and surfaces have been confirmed by observation of infection with the virus in family members as well as health and medical staff (8, 9). According to recent research, the main route of transmission of the virus is through inhalation of infected respiratory droplets with the infected person or contact with the patient's secretions. In air borne transmission, infected airborne droplets are spread through the sneezing or coughing of the infected person into the environment and surfaces in the mouth or nose of people close to the patient and then transported into the lungs (10). There is also the possibility of a person becoming infected with the novel coronavirus through contaminated objects or surfaces. After contact with the infected surface and then touching the mouth, nose and eyes with infected hands, the virus enters the body through the mucosa (11). In addition, the presence of the novel coronavirus in the feces and municipal sewage system has been proven (12). Already, transmission of the virus by the domestic insects living in and around humans is a scientific hypothesis. Domestic insects including houseflies and American cockroaches often live in the municipal sewage system and feed on fecal matter. These insects are able to transmit microbes mechanically from the sewage system to human's dwellings (13).

Considering that Iran is one of the major foci of SARS-CoV-2 infection (14) and for the first time in Iran on 19 February 2020, two patients infected by the novel coronavirus were reported from Qom in Kamkar-Arabnia Hospital as a center for infectious diseases and patients with COVID-19; this study was aimed to molecular evaluation of the novel coronavirus infection isolated on external and internal surfaces of cockroaches and flies collected from Kamkar-Arabnia Hospital in Qom City, Iran.

Materials and Methods

Study area

The study was done in Kamkar-Arabnia Hospital (within the latitudes and the longitudes 34.646765°N, 50.886532°E) in Qom City, Iran. Qom city is located in central Iran within the latitudes and the longitudes of 34.15°-35.15° N and 50.30°-51.30° E, respectively. At the same time, as in other countries, the Covid-19 infection occurred in Iran and Qom city was one of the areas of the country where the first cases of this disease were reported (14). Patients with the coronavirus infection were referred to Kamkar-Arabnia Hospital as a center for infectious diseases and patients with Covid-19 (Fig. 1).

Data collection

In the cross-sectional study, the sampling process on American cockroaches and

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houseflies was done at treatment and sick rooms, kitchens, toilets, and bathrooms in August 2020. The hand catch was done using sterile entomological forceps and sticky traps for American cockroaches' specimens. In the sticky trap method, a tile or plastic sheet with approximate dimensions of 20* 20 cm was used. A small amount of absorbent material, such as bread soaked in beer or a small piece of cake, was placed on it as bait, and a circle of non-dry glue known as mouse glue was placed around it. Cockroaches were trapped in these traps. In the hand catch method, after observing American cockroaches, they were taken and placed in separate sterile tubes using sterile gloves, and then transferred to the medical entomology laboratory of Qom University of Medical Sciences. In addition, the hand net was used for collecting house flies. In order to detect probable infection to the novel coronavirus in insects, sampling was performed from the external surfaces of them. Then, the external surface of each sample was thoroughly washed with 5 ml of sterile distilled water and rinsed with 70% ethyl alcohol for 2 minutes to remove contaminants from the external surface of their bodies. To eliminate the effects of alcohol on the samples, the samples were then placed in sterile physiological saline for 3 minutes. Then the internal surface of captured cockroaches and houseflies was labeled in separate tubes. The samples were stored at 4-80C in the lab of a knowledge base company for routine molecular analysis on coronavirus (DSPR, Qom, Iran).

Data analysis

Bioinformatics analysis and primer selection

The optimization and setting up of multiplex one-step qPCR technique for the detection of different gene regions of SARS- CoV-2 depends on the selection or design of multiplex primers and probe in order to screening and definitive diagnosis. For this purpose, the complete genomes of this virus were obtained from Gene Bank. After aligned the sequences by CLC genomics workbench 12 (CLC, Bio-QIAGEN, Aarhus, Denmark), the E gene and RdRp gene were considered for amplifying. The evaluated primers were selected for the multiplex one-step-qPCR method [15]. (Table1).

Non-competitive internal control design

To achieve the goal of building internal control, an innovative gene structure was designed and synthesized. This gene structure contains 76 bases; 23 bases at the end of 3 placed as a specific reverse primer for the COII gene. The remaining 56 bases were designed as a stem-loop which denaturant at 600C and is amplified with a primer and probe on the HEX channel (Fig.2). Exogenous heterologous internal control could be added as amplification control in the extraction step and investigation of this process.

RNA extraction

Coronaviruses RNA was extracted using Viral DNA/RNA Extraction Kit (Padtan Gostar Isar, Tehran, Iran) according to a modified manufacture protocol. Before the extraction process, the samples must reach room temperature. At the first 500 µl Lysis Buffer added to a 1.5 ml nuclease-free tube. Then 4 µl exogenous heterologous internal control along with 200 µl of the prepared sample was added to the tube and done pulse vortexing for 15 seconds. After the addition of 200 µL of Precipitation Buffer, the lysate was transferred to the binding columns for RNA isolation. Finally, the columns were washed and genomic RNA eluted by 50 mL prewarmed (60ºC) DNase-RNase free water. An approved positive and negative control was extracted simultaneously with other samples.

The quantity and quality of RNA extracted was determined by Nanodrop One

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Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the gene related to internal control, respectively.

Multiplex one-step Taq-Man qPCR optimization

Multiplex one-step qPCR amplification was carried out in Rotor-Gene 6000 instrument (Corbett Research, Sydney, Australia) to the detection of the novel coronavirus in three channels of FAM, ROX, and HEX for isolation of RdRp gene, E gene, and internal control receptivity, using the following protocol: 4 µL 5x qPCR Probe Mix (Solis Bio Byne, Estonia), 0.5µL Reverse transcriptase enzyme (Solis Bio Byne, Estonia), 7 µL of 10 pmol each three pair primers and probes, and 8.5 µL of RNAin 20 µL final reaction volume. The amplification condition was optimized by incubating the reaction mixtures at 50˚C for 25 min (Revers transcription), 95˚C for 10 min (Initial activation), followed by 45 cycles at 94˚C for 15 s and 58˚C for 60 s. All the samples were investigated in duplicate, and novel coronavirus positive samples were detected by analyzing the amplification curve on all three channels simultaneously.

Results

A total of 18 specimens were collected in this study, of which 12 specimens were related to the American cockroaches and 6 specimens to the houseflies.

The results of the evaluation of the insect samples using the multiplex one-step qPCR technique in terms of infection with the novel coronavirus were analyzed according to Table 2.

The internal control gene amplification plot,

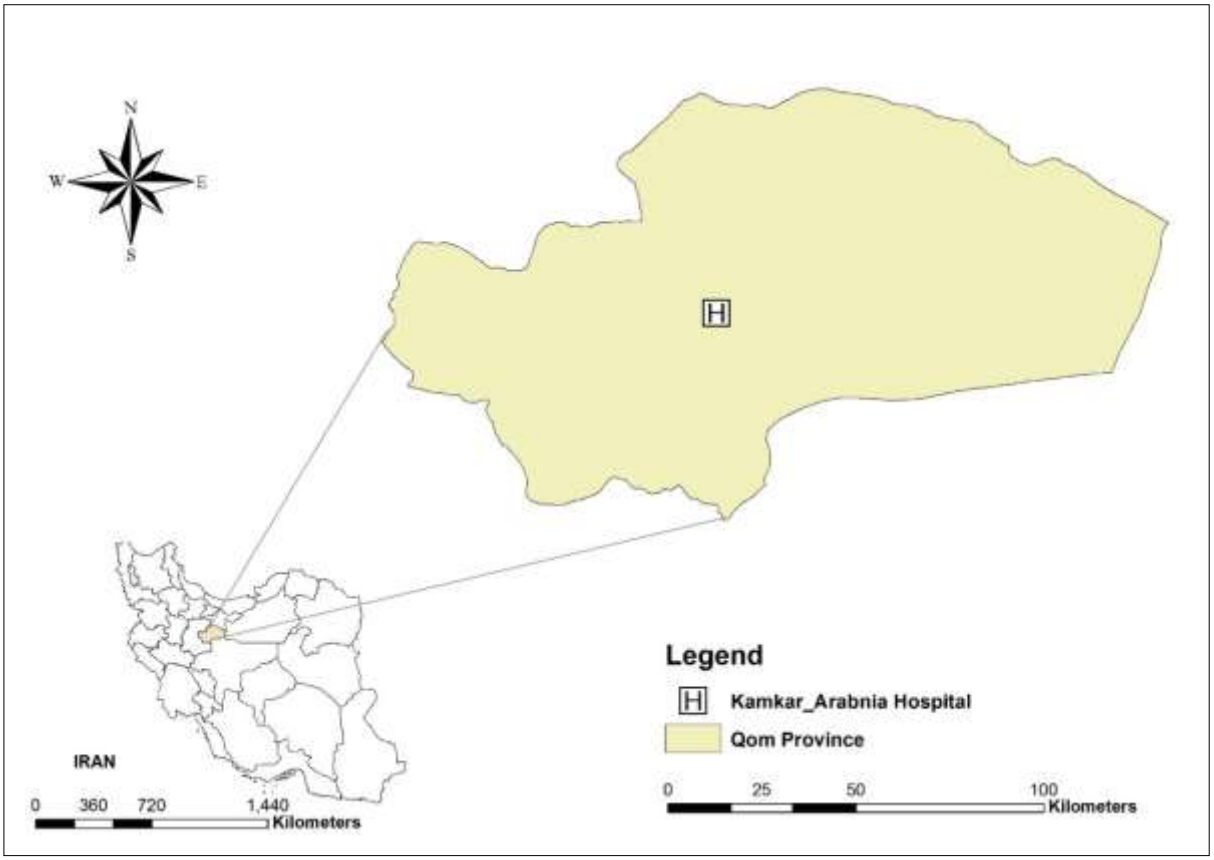

Fig. 1. The study area; Kamkar-Arabnia Hospital in Qom City, Central Iran

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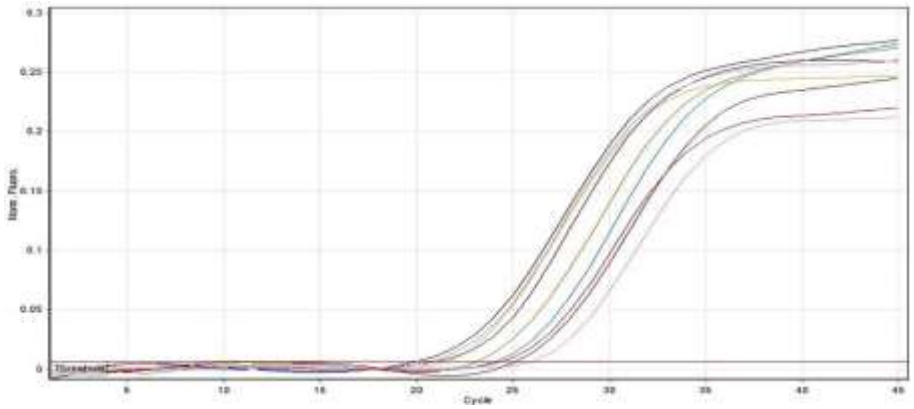

Fig. 2. Amplification plot of RNase P gene on HEX channel of Rotor-Gene 6000 instrument on insect specimens using multiplex one-step qPCR along with positive and negative control

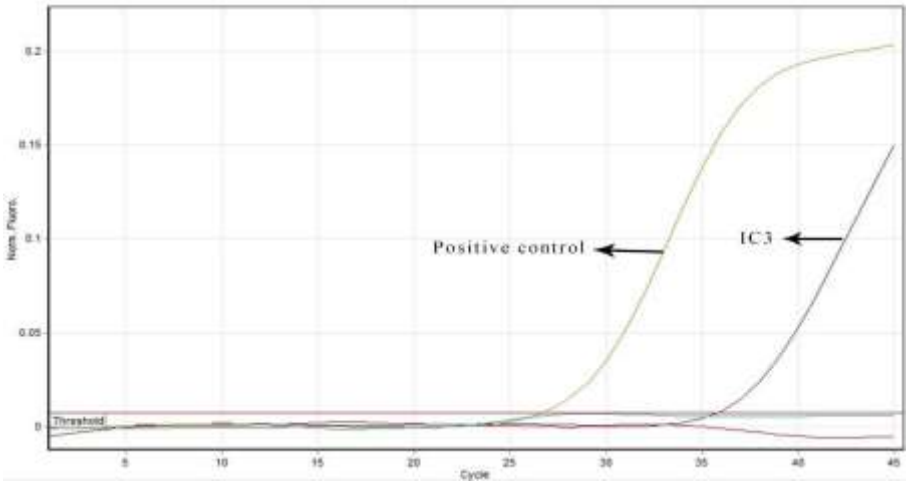

Fig. 3. Amplification plot of E gene on ROX channel of Rotor-Gene 6000 instrument on insect specimens using multiplex one-step qPCR along with positive and negative control

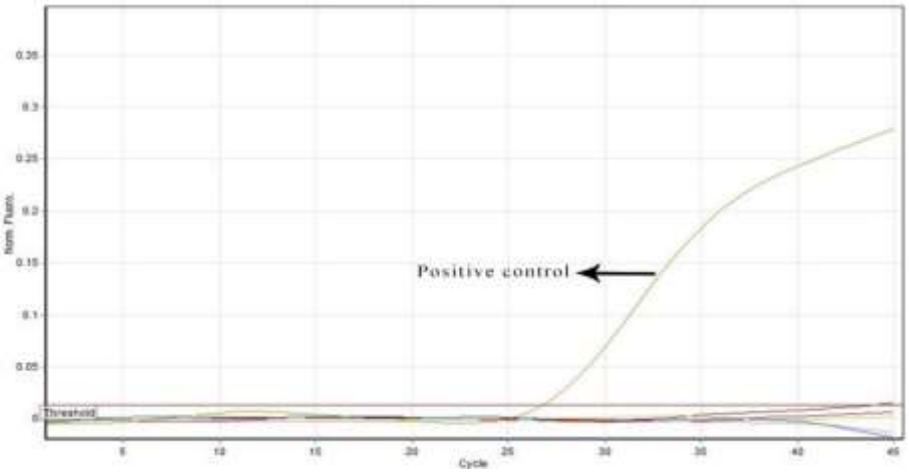

Fig. 4. Amplification plot of RdRp gene on FAM channel of Rotor-Gene 6000 instrument on insect specimens using multiplex one-step qPCR along with positive and negative control

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Table 1. Primers and probes for detection novel coronavirus

Primer name	Reference
E_Sarbeco_P1	ROX-ACACTAGCCATCCTTACTGCGCTTCG-BBQ
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA
RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ
RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG
RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA
RP-probe	HEX-CGCAGAGCCTTCAGGTCAGAACCCGC-BHQ
RP-F	TGGCGGTGTTTGCAGATTTGG
RF-R	TGAGCGGCTGTCTCCACAAG

Table 2. Analyzing the results of examining all three genes E, RdRp, and RNase P using the multiplex one-step qPCR technique

Row	Positive control	Negative control	HEX (IC)	FAM (RdRp)	ROX (E)	Result
1	<35	>40	<45	>40	<39	SarbecoV Positive
2	<35	>40	<45	<39	<39	2019-nCov Positive
3	<35	>40	<30	>40	>40	detectable Not
4	<35	>40	<45	<39	>40	Repeat Test***
5	<35	<35	<35	+/-	+/-	Contamination Repeat
6	>35	>35	>45	+/-	+/-	PCR failure Repeat

which was evaluated of the extraction and qPCR process in this study, for all samples had a CT (Cycle of Threshold) between 22.21-26.20 (Fig.3).

Investigation of coronavirus E gene using the multiplex one-step qPCR technique on the collected samples showed an amplification plot in CT= 35.70 related to (Cockroach Internal sample 3) CI3. This sample belonged to the internal surfaces (digestive system) of American cockroaches collected from the treatment and sick room of Kamkar-Arabnia Hospital, Qom, Iran. In the RdRp gene analysis, no amplification plot was observed in any sample except for positive control (Fig.4).

Discussion

Respiratory diseases are usually transmitted through respiratory droplets (16). In the case of the Covid-19 infection in China and other countries, first most patients were infected with respiratory droplets and contact routes (17). Recently, in addition, other ways of transmitting the coronavirus have been mentioned, including airborne transmission (16). It should declare that droplet transmission takes place when an individual is in close contact with a person who has respiratory infection symptoms related to Covid-19 infection. Also, airborne transmission of the novel coronavirus related to the presence of the virus within droplet nuclei, which are commonly determined to be particles <5μm in diameter, may remain in the air circulation for long times and be transmitted to other people over distances more than 1 meter (18). Furthermore, there is some document that Covid-19 virus can lead to intestinal infection in humans and be present in feces. So, to date some studies have detected the novel coronavirus from sewerage system and feces (12). Besides that, it has been proven that domestic insects such as American cockroaches (*Periplaneta americana*) and houseflies (*Musca domestica*) live in the sewage system and they are the mechanical vectors of microbes and parasites such as viruses,

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bacteria, and parasites such as amoebae, *Shigella* infection (Shigellosis), helminthes eggs to humans (19-21). In the present study, the theory of mechanical transmission of the novel coronavirus by insects from the sewage system was proposed and a specimen of the digestive system of American cockroaches was positive for the coronavirus in the E gene. Findings of other study confirm that the clinical samples with E only amplification should not be dismissed as non-specific results. Colton, Hayley, et al found that 85% of E only samples were confirmed by a second assay targeting the N gene or an alternative RdRp only assay. Furthermore, we observed a mean difference of three CT when comparing the E gene to RdRp values, which may suggest the possibility of higher copy numbers of the E gene (22). The unique transcription strategy of the coronaviruses, in which genes towards the 3' end of the genome would be present in higher copy numbers during active viral replication, could explain these claims (23). Given this hypothesis and the fact that the virus load in the CI3 sample was low, the E gene in CT=35.7 showed a curve. The lack of curve in the RdRp gene is justified. In addition, amplification of innovative internal control designed in this study to prevent the false-negative due outcome from inhibitor interference could sure use the proper performance of the nucleic acid extraction method. These domestic insects are usually found indoors in the living environment and workplace and human services, including hospitals in various wards (24). Due to the feeding of these insects from the sewage environment, microbial contamination such as coronavirus is carried out by the external organs of the insect body such as mouth appendages, body surface hairs, legs and wings. These microbes can be transmitted to people by re-feeding from the hospital environment and patients and their companions or are hospitalized in the wards (20). Furthermore, American cockroaches and houseflies are omnivorous, and in addition to transmitting the coronavirus through the feces and sewage system, they may become infected with the coronavirus by sitting on infected material in hospitals and transmitting it to others.

Conclusion

In the case of the Covid-19 pandemic, due to the close relationship of these insects with humans and their large populations, and their high reproducibility and rapid movement from one place to another, they can be potentially dangerous in relation to the transmission of the coronavirus. It seems necessary to control the population of these insects in central Covid-19 infection hospitals in order to prevent the transmission of the corona virus by insects. In addition to health and treatment measures for patients with Covid-19 infection, it is recommended taking special measures to control the insect population.

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Conflict of interest statement

Authors declare that there is no conflict of interest.

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