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ORIGINAL ARTICLE

First molecular detection of SARS-CoV-2 virus in cockroaches

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Abstract

Coronavirus is one of the main pathogens that primarily targets the human respiratory system. There are several ways to transmit this virus, such as direct contact or droplets spread by coughing or sneezing, and direct contact with fomites and surfaces is another way. This cross-sectional study was conducted in Shiraz, southern Iran, in 2021. 5 locations, including 3 hospitals and 2 dormitories, were selected for the survey. The cockroaches were collected from selected locations and transferred to the Laboratory of Medical Entomology at Shiraz University of Medical Sciences. All specimens were identified morphologically. The external and gastrointestinal washouts of collected samples with sterile phosphate-buffered saline separately were used for molecular analysis. An RT-qPCR assay, which suggests the possible insect-borne transmission, was used. External and gastrointestinal washout of *B. germanica* from Dastgheyb Dormitory and *P. americana* from Ali-Asghar Hospital were positive for contamination with the SARS-CoV-2. Cockroaches spread the virus in the environment and contaminate human food and various surfaces of buildings. Their role will be more important in crowded places such as hotels, lodging houses, restaurants, and hospitals; vector control programs should be carried out with more accuracy in such places.

Keywords Coronavirus · Insect · Real-time RT-qPCR · Vectors · Cockroaches · Iran

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Abbreviations

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
RT-qPCR	Reverse transcription quantitative real-time Polymerase chain reaction
COVID-19	Coronavirus disease of 2019
VTM	Viral transport medium
PBS	Phosphate-buffered saline

Background

Coronavirus is one of the main pathogens that primarily targets the human respiratory system. The first case report of Covid-19 was reported in Wuhan, Hubei Province, China, on December 31, 2019. In late December 2019, several patients were hospitalized with an initial diagnosis of pneumonia of an unknown cause. The patients were epidemiologically linked to China's marine animal wholesale market (Rothan and Byrareddy 2020). The disease spread rapidly to other countries, causing a crisis that was finally declared a pandemic by the World Health Organization, and now

almost all countries of the world are more or less affected by this disease (Yang et al. 2020).

High diffusion power, relatively high lethality, global spread, lack of previous immunity in humans, and lack of vaccine and definitive treatment are the reasons for the importance of this disease. In Iran, the first definite case of the disease was reported in Qom on February 15, 2020, and despite all the measures taken, the disease spread rapidly in other parts of the country. The first case identified in Fars province was a 61-year-old housewife of Iranian nationality. The onset of symptoms was February 18, 2020, and she was referred to the medical center on the same day and was hospitalized in isolation on the same day. Her sampling date was February 22, 2020. During the two weeks before symptoms, she declared her travel history and did not have concomitant factors such as underlying disease and immunodeficiency. His test result has been declared positive, and his final condition is "recovered."

Numerous reports suggest that person-to-person transmission is a route for spreading COVID-19 infection. Person-to-person transmission occurs primarily through direct contact or droplets spread by coughing or sneezing from an infected person (Barrantes 2021; Castaño et al. 2021; Kwon et al. 2021; Liu et al. 2021). Symptomatic patients with coronavirus are the main distributors, but asymptomatic patients should not be underestimated. Current data show that the essential transport routes are droplet transport, contact transport, and aerosol transport. Stool and oral transmission cannot be ignored because SARS-CoV-2 nuclear acid has been detected in patient fecal samples from the United States and China (Barrantes 2021; Castaño et al. 2021; Kwon et al. 2021; Liu et al. 2021; Wu et al. 2020; Xiao et al. 2020). New investigations on the virus spread are carried out, and it was reported that the flies could help the virus spread on the fomites and to the human (Balaraman et al. 2021a; Dehghani and Kassiri 2020; Soltani et al. 2021).

Insect order is the most populous animal group with economic, medical, and veterinary importance. There has been a relationship and partnership between human health and some insects for a long time (Dent and Binks 2020). However, in the nineteenth century, insects and other arthropods' role in transmitting certain diseases to humans was established. Among these insects, cockroaches always affect human health and can be the vector of pathogenic bacteria and viruses and an intermediary host for intestinal worms and pathogenic protozoa. They are one of the most important vectors in transmitting and spreading many human diseases. These Pathogens can live in Cockroaches' internal and external body parts for several days, and they can and do distribute them. Contacts with the Cockroaches` external body or Feces to the Substances that humans deal with can lead to the transmission of these diseases (Patel et al. 2022; Roth and Willis 1957; Vahabi et al. 2007).

Cockroaches belong to the Arthropoda phylum, Insecta class, and Blattodea or Blattaria order. Blatta, in Latin, means a light-shunning insect (Hashemi-Aghdam and Oshaghi 2015). More than 4300 species of cockroaches have been described worldwide, belonging to more than 500 genera, and more than 30 species are anthropophilic and associated with human dwellings and their food or waste, but the most widesprate and anthoponotic spacies are *Blattella germanica* and *Periplaneta Americana* (Lee et al. 2021).

The German cockroach is one of the most common cockroaches in the human habitat. They are small in adulthood and live mostly in wooden and metal cabinets and shelves in kitchens. Males are light yellowish-brown, and females are darker than males. This cockroach lives almost everywhere as long as there is shelter, food, and water. *B. germanica* is the most synanthropic cockroach, lives in human dwellings, and is in direct contact with kitchen surfaces, food containers, and human foods (Daniel et al. 1992; Robinson 2005; Scott 1963).

The American cockroach, the eggs of these cockroaches, hatch in favorable weather conditions after 70 days. They are large in adulthood. Both sexes are dark brown and live in sewage. Both males and females have developed wings and can fly (Bell 1982; Brenner and Kramer 2019; Cochran and Organization 1999).

These two species were chosen for the study due to their global distribution and for living in human dwellings and environments.

Methods

Study area

This cross-sectional study was conducted in southern Iran in the capital city of Fars province, Shiraz. Its latitude is about 1500 m above sea level. It has a subtropical, hot, semi-arid climate with a mean annual temperature of 18 °C, relative humidity of 41%, and annual precipitation of 337.8 mm. Ali-Asghar (29°37'32.6"N 52°32'30.5"E) and Shahid-Chamran (29°39'44.7"N 52°29'23.7"E) hospitals are two main centers for Covid-19 positive patient treating hospitals in Shiraz. Fatemeh Zahra Clinic (29°38'10.8"N 52°27'37.8"E) is one of the city's most essential and crowded clinics. Shahid-Dastgheyb Dormitory (29°38'36.1"N 52°30'44.9"E) and Golestan Dormitory (29°38'07.2"N 52°30'49.2"E) are two critical dormitories in Shiraz University of Medical Sciences in which students of medical sciences are living, and these students are training in Hospitals of Shiraz. These

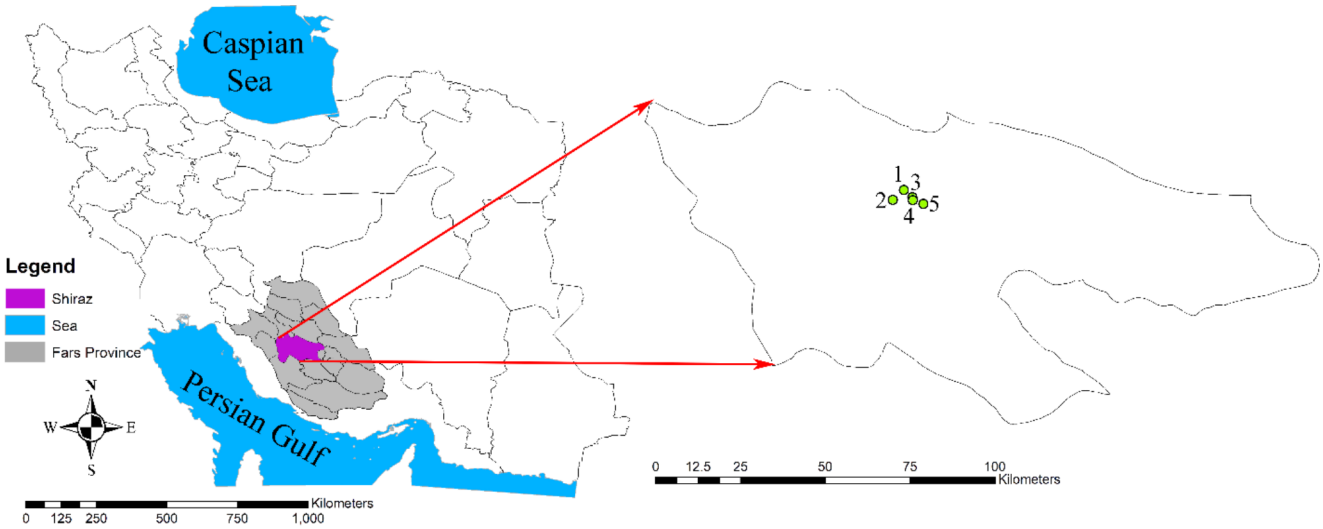

Fig. 1 Sampling locations in Shiraz county (capital of Fars province, south of Iran), Ali-Asghar (1) and Shahid-Chamran (2) hospitals. Fatemeh Zahra Clinic (3). Shahid-Dastgheyb Dormitory (4) and Golestan Dormitory (5)

five locations were selected to survey the contamination of cockroaches to the Covid-19 virus (Fig. 1).

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Collection and identification of cockroaches

Two trained entomologists collected cockroaches from the outsides and insides of buildings in sampling locations for two months of June and July 2021. American cockroaches were collected mainly from the sewage system of buildings, and German cockroaches from inside the buildings, mainly from the kitchens and rooms. According to similar studies on cockroaches' contamination with microbial pathogens, at least 378 samples are needed for molecular assay and analysis (Doroodgar et al. 2005).

All samples spacially *B. germanica* species were collected by hand catch and were separately placed in one streel glass vial (Fig. 2). The cockroaches were transferred alive to the Medical Entomology Laboratory of Shiraz Health School. All the necessary control measures were taken to prevent the contamination of the samplers in the field as well as secondary viral infections of samples during sampling and transferring to the laboratory. Collected specimens were placed in a freezer to kill and were identified morphologically using valid systematic keys, then transferred to a -70° C freezer until molecular analysis.

Molecular experiments

Primer design

Forward (5'CTAACATGCTTAGGATAATGG 3') and Reveres primers (5'CAGGTAAGCGTAAAACTCATC 3') were designed using bioinformatics software such as Gene Runner 4. and Oligo 7.0.

Samples preparation

For sample preparation, 5 samples of the same species were pooled for DNA extraction and PCR assay in each location, to prepare the collected samples, 10 mL of sterile Viral transport medium (VTM) based phosphate-buffered saline was added for every pooled cockroach, placed into a 50 mL falcon tube to washout the external surface of cockroaches. The gastrointestinal tract of the samples was removed and cut lengthwise, and the inner part was washed with 10 mL PBS.

RNA extraction

200 µL of each sample **preparation** supernatant was used for RNA extraction using BehPrep virus nucleic acid extraction kits (AAAA15044000, Tehran, Iran) based on company instructions.

cDNA synthesis

According to the company's protocol, 2 µL of total RNA (100-1000ng) was used as a template in reaction for the synthesis of cDNA by a kit that has been purchased from

Fig. 2 The Sampling of cockroaches using the hand-catch technique (A). Transferring alive to the laboratory (B). Washing out the external body with PBS (C). Washing gastrointestinal tube with PBS (D)

Bioneer Company, Korea (AccuPower® CycleScript RT Premix with (dN6).

Real-time polymerase chain reaction (RT-PCR)

The extracted nucleic acids from each of the samples were amplified using reverse transcriptase Taq-Man RT-PCR using Pishtaz commercial kits (Pishtaz Teb PT. COVID.100, Tehran, Iran).

The SARS-CoV-2 PCR assay consists of two pair primer/probe sets that amplify viral RdRp-gene (labeled with FAM) and N-gene (labeled with HEX) and detect the human RNase P (labeled with ROX) gene as an internal control. A negative (no template) control was used to exclude the possibility of sample contamination during the assay run. A positive template control was also used to verify that the assay run was performed as intended on each assay plate tested. Each 20 µL reaction mixture contained 10 µL master mix and 10 µL extracted genome as RNA template. For the detection of the SARS-CoV-2 genome, ABI StepOnePlus™ Real-Time PCR System (USA) was used under the following thermocycling conditions: 10 min at 55 °C for RT, 3 min at 95 °C for polymerase activation, and then 40 cycles of 15 s at 95 °C and 40 s at 60 °C.

Results

Detection of SARS-CoV-2 by the RT-qPCR assay was considered from a total of 500 cockroaches (250 *B. germanica*, 250 *P. americana*) (Table 1). The presence of the virus was confirmed using external body samples and gastrointestinal tract samples.

Table 1 Number of collected samples from different locations

Location	Species	Total Number	Sampling Places		
			kitchen	Rooms	Sewerage
Dastgheyb Dormitory	*P. americana*	100	0	0	100
	B. germanica	100	90	10	0
Fatemeh Zahra Clinic	*P. americana*	100	0	2	98
	B. germanica	100	97	3	0
Ali-Asghar Hospital	*P. americana*	100	0	0	100
	B. germanica	100	100	0	0
Shahid-Chamran Hospital	*P. americana*	100	0	4	96
	B. germanica	100	95	5	0
Golestan Dormitory	*P. americana*	100	0	5	95
	B. germanica	100	93	7	0

Table 2 The results of RT-qPCR tests, Virus Load, samples number, and the sampling location and contaminated samples

Location	Species	Tested samples	Positive samples	Average RT-qPCR Test & Virus Load per Sample	
				Outer Layer of Samples	Inside the Gastrointestinal tract
Dastgheyb Dormitory	*P. americana*	50	0	0	0
	B. germanica	50	25	≈ 2000 copy/mL lysate	≈ ≤200 copy/mL lysate
Fatemeh Zahra Clinic	*P. americana*	50	0	0	0
	B. germanica	50	0	0	0
Ali-Asghar Hospital	*P. americana*	50	35	≈ 3500 copy/mL lysate	≈ 2000 copy/mL lysate
	B. germanica	50	0	0	0
Shahid-Chamran Hospital	*P. americana*	50	0	0	0
	B. germanica	50	0	0	0
Golestan Dormitory	*P. americana*	50	0	0	0
	B. germanica	50	0	0	0

Samples from 2 locations were positive, 50% of tested *B. germanica* from Dastgheyb Dormitory and 70% of tested *P. americana* from Ali-Asghar Hospital. Of all the cockroaches examined, *B. germanica* samples from Dastgheyb Dormitory and *P. americana* from Ali-Asghar Hospital were positive for contamination with the SARS-CoV-2 RNA virus of varying viral load externally. In addition, the gastrointestinal samples of *B. germanica* from Dastgheyb Dormitory and *P. americana* from Ali-Asghar Hospital were positive for the new SARS-CoV-2 but with less Virus load than samples from the body surface (Table 2).

Discussion

Respiratory coronaviruses, like rhinoviruses, can spread through direct contact with contaminated secretions or large aerosol droplets (Gomes 2020; Reed 1984). In addition, infected individuals can contaminate the surfaces they come into contact with, including many household goods and house appliances (Gomes 2020). Preliminary research shows that coronaviruses, including the SARS-CoV-2 RNA virus, can remain on the surface for hours to days; This can vary depending on different circumstances, for example, surface type, environmental temperature, and humidity (Casanova et al. 2010; Farnoosh et al. 2020; Kampf et al. 2020). A new study presented real-time RTPCR findings of fecal and respiratory samples from cases with SARS-CoV-2 in China showed respiratory samples remained positive for SARS-CoV-2 RNA for an average of 16.7 days, of the 41 (55%) of 74 cases and fecal samples remained positive for an average of 27.9 days after the first symptom start (Wu et al. 2020).

The laboratory transmission of turkey Coronavirus becomes evaluated with the aid of a species of beetle referred to as *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). This study showed that turkey coronavirus could be transmitted mechanically with this insect (Watson et al. 2000).

Another study evaluates biological and mechanical transmission of the Reticuloendotheliosis virus by *Culex pipiens* and *Musca domestica* in vitro and in the field. The results showed that the mosquitoes had the virus in their bodies for up to 5 h, and houseflies survived the virus for up to 72 h. Although all field-collected samples were free of virus, this study shows houseflies are a possible mechanical vector of the virus (Davidson and Braverman 2005).

In another study in 2020, two of 13 flies (one housefly and one drosophila), as well as the mosquito pool of two individuals (not identified), tested positive for African swine fever virus DNA (ASFV-DNA) (Herm et al. 2020). It was previously shown that blood-sucking flies, such as stable flies, can transmit ASFV. It has also been experimentally demonstrated that stable flies can transmit ASFV by biting

(Mellor et al. 1987) or through ingestion by pigs (Olesen et al. 2018).

An overview paper published in 2021 has been declared that contacting or feeding on human feces by an organism such as cockroaches may support SARS-CoV-2 transmission (Sharawi 2021).

In another study, the role of the American cockroach, *Periplaneta americana*, as a mechanical vector for *Eimeria tenella* was evaluated, and *E. tenella* oocysts were found in the digestive tract and feces of infected cockroaches up to day 4 after ingestion of oocysts. Oocysts recovered from the digestive tract and feces of cockroaches remained infective for 4 and 3 days for chickens, respectively (Jarujareet et al. 2019).

Another study in Thailand evaluates the in vitro transmission of the avian influenza virus (HPAI) subtype H5N1. 1500 adult flies were used in three groups. Group A had no contact with the virus, but groups B and C were exposed to the virus with food for 15 min. Group B was immediately homogenized and given to sensitive chickens. Group C was homogenized after 24 h. The results showed group B and C intervention died, although the load of the virus was more in group B. This study showed that houseflies could mechanically transmit this viral disease among birds (Wanaratana et al. 2013). Another study in Japan showed blowflies could mechanically transmit H5N1 bird flu (Sawabe et al. 2011).

In a recent study in Iran, the in vitro infestation of the avian influenza virus (AIV) subtype H9N2 by houseflies was tested; on the body surface, the virus could survive up to 24 h and up to 96 h in internal tissues. It indicates that the houseflies could be a mechanical vector of the avian influenza virus (Salamatian et al. 2020).

Recently, the potential of house flies to mechanically transmit SARS-CoV-2 was investigated, and the results indicate, under laboratory conditions, the SARS-CoV-2 can live in house flies for up to 24 h. They showed that house flies could mechanically transmit SARS-CoV-2 genomic RNA to the environment for up to 24 h (Balaraman et al. 2021b). Another study on field-collected house flies in Shiraz demonstrates that collected flies from Corona Treatment Centers are infected with the virus and can spread the virus in the environment (Soltani et al. 2021).

A recently conducted study in Italy showed that SARS-CoV-2 could be detected in the particulate matter carried by honey bee foragers, in this study foraging honey bees were positive for the target genes of viral SARS-CoV-2 RNA when coming back to the colony (Cilia et al. 2022).

Conclusion

Cockroaches are among the most prevalent insects globally, and they are cosmopolitan; due to their gregarious nature and synanthropic preferences, cockroaches are commonly found in or near human dwellings. They are also indiscriminate feeders that disseminate microbial pathogens from waste-contaminated breeding habitats to human foods, which human hosts may ingest. The SARS-CoV-2 contamination causes intense gastrointestinal infection in a few people, and the virus is detected in their feces. The viability of the virus has been reported for up to 72 h on some surfaces and feces and urine. Feces can be considered an essential source of SARS-COV-2 infection. Therefore, all organisms that contact it or eat it could be associated with SARS-CoV-2 transmission. Thus, the role of insects such as houseflies and cockroaches in SARS-CoV-2 infections could be essential (Fig. 3). It seems that the virus can live on cockroaches because of their habitat condition. They usually live in highly humid places. The role of these insects in the spread of SARS-CoV-2 will be more important in crowded places such as hotels, lodging houses, restaurants, and hospitals.

To mechanically transmit the virus, cockroaches must first obtain the virus from the contaminated source, ingest a sufficient amount of the virus, and colonize it on the crop, gut, or body surface. Each virus needs to be stable and viable during this period. Overall, this study has shown that cockroaches can acquire, and contaminate the SARS-CoV-2 virus in the environment.

Transmission of the virus by cockroaches can make the epidemiology of the disease very difficult; therefore, the role of vector control, along with methods of prevention and control of other diseases, becomes essential. Healthcare professionals plan and implement Integrated Vector Management (IVM) programs in high-risk areas during the warmer months of temperate regions and every month of the year in tropical areas where the population and activity of these insects increase. Further in vivo laboratory and field studies are needed to investigate the exact role these insects play in the circulation of this deadly universal virus.

The presence of Covid-19 in cockroaches can be very important, opening new frontiers to investigate the environmental diffusion of air, particulate matter, water and sewers as it is reported from other insects such as honey bees and house flies. Monitoring the presence of the SARS-COV-2 virus in insects can help the determination of the epidemiological extent of the disease in the community.

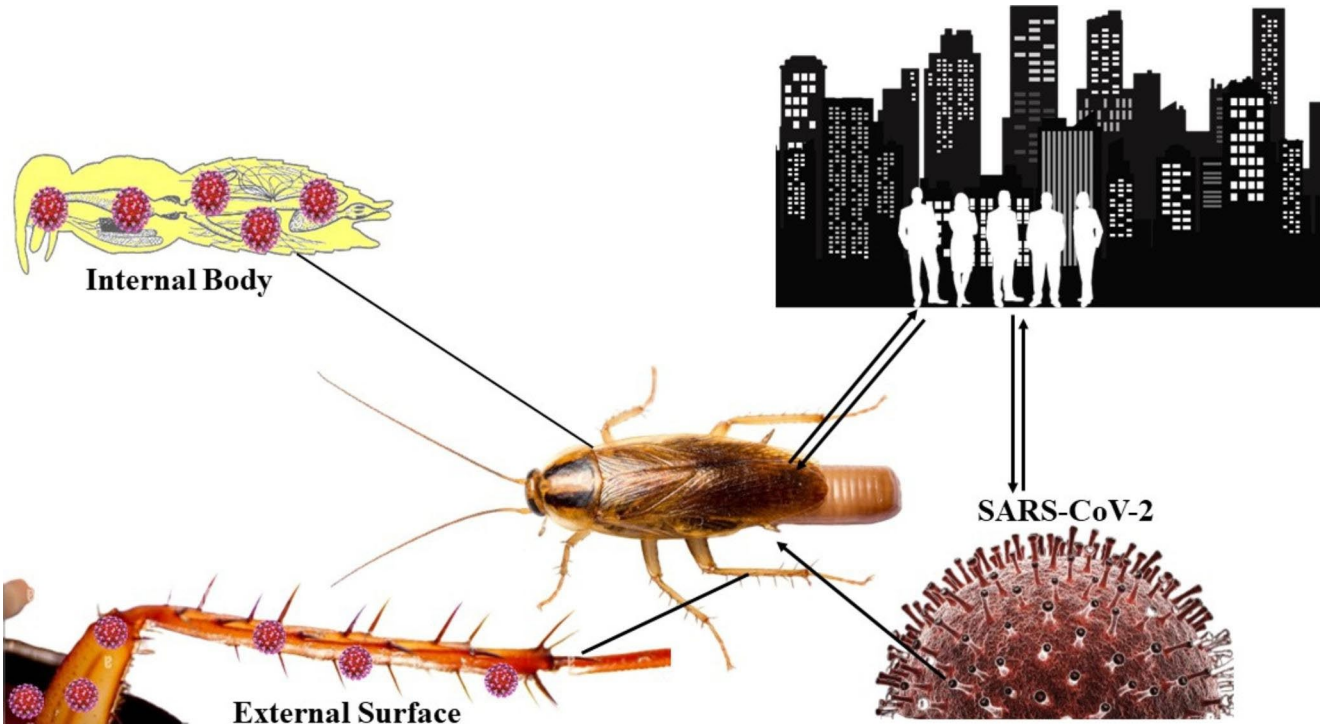

Fig. 3 Molecular detection of SARS-CoV-2 in field-collected cockroaches

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Authors' contributions MV conceived the paper, analyzed the results, and wrote the first and final version of the manuscript. AH, MM, NA, HA, HA, HR supported data collection. KA and MK supported the interpretation of results and revision of the manuscript. MJ does the molecular analysis. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate This study was approved by the Shiraz University of Medical Sciences ethic committee (IR.SUMS.REC.1399.997).

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

Financial interests The authors declare they have no financial interests.

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