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ORIGINAL ARTICLE

Giardia lamblia assemblages A and B isolated from symptomatic and asymptomatic persons in Hamadan, west of Iran

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Abstract *Giardia* is a very abundant organism bringing about diarrhoea in human beings. The focus of this analysis was the detection of *Giardia lamblia* assemblages in human stool specimens in Hamadan, west of Iran, as well as the association between obtained assemblages and clinical symptoms. Faecal samples of 4066 individuals admitted to the medical and health care facilities in Hamadan were inspected microscopically for the existence of *Giardia* cysts/trophozoites, and the clinical symptoms of the patients were recorded. The DNA of positive samples was isolated from and the nucleotide sequences of both glutamate dehydrogenase (*gdh*) (n = 15) and triose phosphate isomerase (*tpi*) (n = 8) genes were analyzed. In direct microscopy, a total of sixty-four samples (1.6%), were considered as positive for *G. lamblia* cysts or trophozoites. The sequence analysis showed that 18 out of 23 sequenced isolates (78.2%) were assemblage A and 5 (21.7%) were assemblage B. Clinical symptoms were observed in 44.4% and 40% of patients with assemblages A and B, respectively. Overall, the predominant assemblage A detected in the tested samples along with bioinformatics analysis suggest a potential zoonotic transmission in the region of the study. Although advanced analyses are necessary to understand the foundation and path of the infection, it seems that more sanitary regulations regarding contact with livestock and pet animals are essential.

Keywords Assemblage A and B · Clinical symptoms · *Giardia lamblia* · Iran

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Introduction

Giardiasis is known as a very common worldwide parasitic infection, which is caused by binucleated flagellated protozoan known as *Giardia*. This parasite lives and reproduces in the small intestine (Lopez-Romero et al. 2015). Giardiasis, as a gastrointestinal disease, has various experimental demonstrations; varying from asymptomatic to severe or prolonged diarrhoea plus weight loss and epigastric pain. Infection begins with the absorption of water or food polluted with cysts of the parasite. Worldwide the number of new cases of the disease is estimated at around 280 million infections per year. This neglected disease could threaten the individual health, and the clinical impact of the disease is stronger in immunodeficient individuals and young children (Ankarklev et al. 2010; Cacciò et al. 2017). Also *Giardia lamblia* infection in early childhood had a strong adverse effect on cognitive function (Ijaz and Rubino, 2012).

Currently, based on evaluating the genetically different housekeeping genes like the triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*), *G. lamblia*, is categorized to eight assemblies of A, B, C, D, E, F, G, and H (Abbas et al. 2016; Gasparinho et al. 2017), all have been found in the intestine of diverse vertebrate hosts. The

assemblage A and B infect individuals and other hosts comprising of cats and dogs. However, others assemblies are found in particular hosts (Cacciò and Ryan 2008; Itagaki et al. 2005).

Results of other studies indicate that the *tpi* gene is reformed better than the *gdh* gene for effectual favoritism amongst the two chief assemblages. Therefore, it can be tremendously advantageous to have discovery techniques to target loci with a high degree of polymorphism like *tpi* when a usual contamination foundation is surely interested (Traub et al. 2004). The 18S rDNA and *tpi* gene is more preserved than the *gdh* gene; this makes it achievable to differentiate amongst the sub-groups, whereas the 18S rDNA and *tpi* gene is more preserved and favoritism within the sub-assemblages is not possible (Bertrand et al. 2005; Coradi et al. 2011). Therefore, the usage of the *gdh* gene may be more reliable to evaluate the aspects of zoonosis transmission (Read et al. 2004; Traub et al. 2004).

Current data suggest that the *G. lamblia* assemblages are different from each other with respect to pathogenicity and clinical presentations (Cacciò and Ryan, 2008). Therefore, the use of molecular approaches is necessary for the determination of the assemblages infecting humans. Based on analysis of single nucleotide polymorphisms and genotyping of β-giardin (bg), 18S rRNA, *gdh*, and *tpi*, A and B assemblages were positioned in five sub-assemblages (e.g. AI, AII, AIII, BIII and BIV) (Rayani et al. 2017). It is notable that sub-assemblages AI, AII, BIII, and BIV are found among humans and animals and are considered as zoonoses, though sub-assemblage AIII exists exclusively in animals (Sprong et al. 2009).

As in the other part of the world, giardiasis is a chief parasitic health difficulty in Iran (Badparva et al. 2014; Bahrami et al. 2017; Nasiri et al. 2009). However, there is no data about the prevalence of different assemblages of *G. lamblia* in a lot of the parts of the nation including Hamadan province. Therefore, this research intended to reveal the prevalence of *G. lamblia* assemblages in Hamadan, the capital city of Hamadan province in west Iran, and their possible relationship with clinical symptoms of the patients.

Materials and methods

The present research was accomplished in agreement with the ethical standards of the Declaration of Helsinki and has been permitted by Hamadan University of Medical Sciences ethics committee (Grant No. 9208072464) with respect to the human rights and a written informed consensus was attained from every participant before gathering data. The study was conducted from July 2014 to September 2015.

Samples

In this research, overall 4066 stool samples were gathered from individuals (2468 male and 1598 female), which were admitted to the medical and health care facilities of Hamadan during 2014 and 2015. Following record of the demographic data and clinical symptoms, fecal samples were collected by patients into sterile plastic containers and as soon as possible transported to the research laboratory of Department of Medical Parasitology and Mycology, School of Medicine, Hamadan University of Medical Sciences, and immediately were examined microscopically for identification of the cyst or trophozoite of *G. lamblia* by using both direct wet mount and formalin-ether sedimentation techniques. Positive stool samples were stored in 70% alcohol at a temperature of 4 °C till DNA extraction.

DNA extraction

Roughly 300 µl of fecal suspension was rinsed three times using distilled water to eliminate drops of alcohol and after the genomic DNA of the isolates was separated directly from faeces with the use of the QIAamp Mini Kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. The extracted DNA was stored in sterilized tubes and frizzed at − 20 °C until tested for PCR.

PCR

The *tpi* genes were amplified with the use of nested PCR by a way already explained (Sulaiman et al. 2003). For *tpi* gene a 530 bp region was amplified with the use of the primers TPIeF (5′-AAATXATGCCTGCTCGTCG-3′) and TPIeR (5′-CAAACCTTXTCCGCAAACC-3′) in the first PCR and TPIiF (5′-CCCTTCATCGGIGGTAACTT-3′) and TPIiR (5′-GTGGCCACCACICCCGTCC-3′) in the secondary PCR. For *gdh*, a 432 bp region was amplified with the use of the primers GDHeF (5′-GACGCATCAACGTCAACC-3′) and GDHeR (5′-GAGCTTCTCGCAAGCAAAC-3′) in the first PCR and GDHiF (5′-CAGTACAACTCTGCTCTCGG-3′) and GDHiR (5′-GTTGTCCTTGCACATCTCC-3′) in the secondary PCR.

For each gene an ultimate volume of 25 µL was utilized to execute the first PCR reaction, which comprised of 15 ρM of each primer, 12.5 µL of 2X PCR kit master mix (Ampliqon ApS, Literbuen 11, DK-2740 Skovlunde, Denmark) and 10 ng of extracted DNA. An ultimate volume of 30 µL was utilized to execute the second PCR reaction, which contained 15 µL of 2X PCR master mix, 15 ρM of each primer, and 10 ng of the first PCR produce. For the PCR assay, amplification was accomplished in a thermocycler (Techne Ltd., Cambridge, UK) and positive and negative controls together were involved in every

round of PCR to certify the outcomes. Then, PCR produces were electrophoresed with agarose gel 1.5%, stained with ethidium bromide, and visualized underneath UV light.

Sequence analysis

The secondary PCR produces of 15 amplified *gdh* and eight *tpi* genes were purified and exposed to forward direct sequencing with the use of a BigDye Terminator cycle sequencing kit (PE Biosystems, Foster City, CA) and a genetic analyzer (Prism^{TM} 3130 $\times$ 1; ABI).

The *gdh* gene analysis was used to evaluate the route of transmission, and the *tpi* gene was employed to determine the assemblages. Nucleotide sequences were edited manually by using the Chromas program version 1.0 and the consensus sequences were presented to the GenBank/EMBL/DDBJ database with the accession numbers KY444769–KY444791. Assemblages were verified with the use of nucleotide Basic Local Alignment Search Tool (BLAST) provided by NCBI (http://www.ncbi.nlm.nih.gov/).

The sequences were aligned using ClustalW2 and a phylogenetic tree was created with the use of MEGA4 software, by the neighbor-joining technique established on evolutionary distances estimated by the Kimura two-parameter model with 1000 bootstrap sampling. Representative sequences of *Giardia* isolates for each animals and human were obtained from the NCBI database and were contained in the tree.

Statistical analysis

SPSS software version 17 was utilized for statistical analysis. The analytical assessment was carried out using Chi square and Fisher's tests and the p value < 0.05 were considered significant.

Results

In this current research, 4066 complete stool samples were inspected using parasitological procedures, from which 64 (1.6%) individuals were positive for *G. lamblia* cysts or trophozoites. The frequency of *G. lamblia* infection and clinical symptoms according to demographic characteristics and some other variables are shown in Table 1.

The outcomes displayed that the occurrence of *Giardia* infection in male (67.18%) was significantly higher than female (32.81%) ($p < 0.001$). Moreover, the most frequency (54.68%) of *Giardia* infection was reported in the 20–39 years old age group ($p = 0.017$). The evaluation of patients' residential area revealed that most (84.37%) of individuals infected with *Giardia* were from urban areas ($p = 0.99$) and 19 (29.69%) of 64 *Giardia*-infected patients had a history of contact with animals ($p = 0.12$).

Because of the financial limitations, we only had 30 sequence budgets samples. So we decided to sequence 15 samples of the *gdh* gene and 15 samples of the *tpi* genes. It is nortworthy that by *gdh* gene all 15 isolates and using *tpi* gene only 8 isolates were successfully sequenced. The results of analysis of *tpi* and *gdh* genes revealed that among 23 isolates, 18 (78.2%) were assemblage A, and five (21.7%) were assemblage B (Table 2). Considering the *tpi* gene, assembly B was indicated in 5 cases while 3 isolates fitted into assemblage A. Using the *gdh* gene all isolates belonged to assembly A.

The *gdh* gene sequencing showed that all isolates belonged to sub-assemblage AII having 99–100 homology with each other and 95–100% homology with the related sequences from animals such as cat (AB569385), sheep (KY083429), and goat (AB692779) and wastewaters (KT235915) in Iran and also from humans in Europe (MG515187), India (JF918514), and Cuba (EU594662), and furthermore from vegetables in Brazil (KJ741314) and Romanian surface waters (KX539310) deposited in GenBank. Figure 1 shows phylogenetic relationships of one sample in this study (KY444781) with some human and animal isolates of *G. lamblia* that previously published in GenBank. Figure 2 shows Phylogenetic relationship among all *G. lamblia* isolates sequenced in this study based on both loci.

According to genotyping for the *tpi*, two individuals (40%) presenting genotype B were symptomatic, while 60% of the people with genotype B were asymptomatic. On the other hand, 33.3% of cases presenting genotype A had symptoms, and 66.7% of people with genotype A had no symptoms. Based on the *gdh* gene, 46.7% and 53.3% of individuals presenting genotype A were symptomatic and asymptomatic, respectively (Table 3).

Discussion

Giardiasis has been recognized as a zoonosis, which transmitted to human by ingesting *G. lamblia* contaminated food and water. It also proposed the most frequent cause of non-bacterial diarrhoea in North America (Lalle 2010). *Giardia* is a very usual source of traveller's diarrhoea in the world. Recently, published data revealed that *G. lamblia*, as a complex species, can be classified into eight (A–H) main assemblages. Studies on the identification of *Giardia* assemblages revealed that only assemblages A and B have been distinguished in human (Abbas et al. 2016; Fantinatti et al. 2016). Also, it is narrated that various assemblages of *G. lamblia* have distinct clinical features and pathogenicity (Cacciò and Ryan 2008). Consequently, the existing

Table 1 The frequency of *G. lamblia* infected patients and clinical symptoms according to some demographic features

Characteristic	No. (%)	Stomachache No. (%)	Flatulence No. (%)	Cachexia No. (%)	Diarrhea No. (%)
Gender					
Male	43 (67.2)	12 (27.9)	9 (20.9)	3 (7.0)	3 (7.0)
Female	21 (32.8)	7 (33.3)	10 (47.6)	2 (9.5)	0 (0)
Age (years)					
0–19	19 (29.7)	10 (52.6)	7 (36.8)	0 (0)	2 (10.5)
20–39	35 (54.7)	6 (17.1)	7 (20.0)	3 (8.6)	1 (2.9)
$\geq$ 40	10 (15.6)	3 (30.0)	5 (50.0)	2 (20.0)	0 (0)
Residential area					
Urban	54 (84.4)	16 (29.6)	17 (31.5)	4 (7.4)	1 (1.9)
Rural	10 (15.6)	3 (30.0)	2 (20.0)	1 (10.0)	2 (20.0)
Animal contact					
Yes	19 (29.7)	7 (36.8)	5 (26.3)	2 (10.5)	0 (0)
No	45 (70.3)	12 (26.7)	14 (31.1)	3 (6.7)	3 (6.7)
Assemblages					
A	18 (78.3)	5 (27.8)	6 (33.3)	1 (5.6)	1 (5.6)
B	5 (21.7)	0 (0)	2 (40.0)	1 (20.0)	0 (0)

Table 2 Genotypes of *Giardia lamblia* isolates determined by sequence analysis of *gdh* and *tpi* genes

Gene	No.	Isolate	Accession no.	Assemblage	Sub-assemblage
tpi	1	GIK1	KY444784	A	AII
	2	GIK2	KY444785	B	Allele
	3	GIK3	KY444786	B	Allele
	4	GIK4	KY444787	A	AII
	5	GIK5	KY444788	A	AII
	6	GIK8	KY444789	B	Allele
	7	GIK9	KY444790	B	Allele
	8	GIK10	KY444791	B	Allele
gdh	1	KGGI1	KY444769	A	AII
	2	KGGI2	KY444770	A	AII
	3	KGGI3	KY444771	A	AII
	4	KGGI4	KY444772	A	AII
	5	KGGI5	KY444773	A	AII
	6	KGGI6	KY444774	A	AII
	7	KGGI7	KY444775	A	AII
	8	KGGI8	KY444776	A	AII
	9	KGGI9	KY444777	A	AII
	10	KGGI10	KY444778	A	AII
	11	KGGI11	KY444779	A	AII
	12	KGGI12	KY444780	A	AII
	13	KGGI13	KY444781	A	AII
	14	KGGI14	KY444782	A	AII
	15	KGGI15	KY444783	A	AII

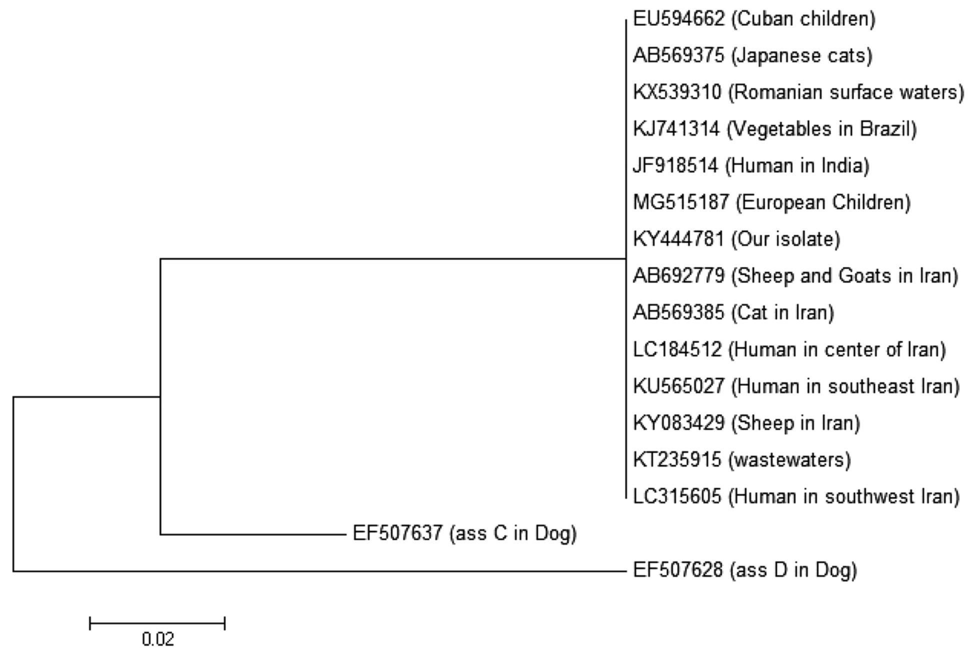

Fig. 1 Phylogenetic relationships of *G. lamblia gdh* gene from different parts of Iran and animal isolates of *G. lamblia* that previously published in GenBank

Table 3 Distribution of *G. lamblia* genotypes according to epidemiological factors

Variable	Category	Frequency	Assemblage	
			A	B
Gender	Male	16	14	2
	Female	7	4	3
Age (years)	0–19	4	3	1
	20–39	12	10	2
	$\geq$ 40	7	5	2
Residential area	Urban	19	15	4
	Rural	4	3	1
Contact with domestic animals	Yes	5	3	2
	No	18	15	3
Clinical features	Symptomatic	10	8	2
	Asymptomatic	13	10	3
Total		23	18	5

research was carried out to distinguish the frequency of the *G. lamblia* genotypes A and B and the relationship of assemblages with the patients' demographic data such as sex, age, residential area and some clinical features in Hamadan city of Iran.

Our results demonstrated that prevalence of giardiasis was 1.6 percent that is similar to those found in Kurdistan (1.66%) (Bahrami et al. 2017), Qazvin (0.85%) (Sadeghi and Borji 2015) and Karaj (3.8%) (Nasiri et al. 2009). In contrast, higher prevalence of giardiasis found in Shahre-Ray (25.8%) (Arani et al. 2008), Kirkuk (10.31%) (Salman et al. 2016) and Shiraz (10.6%) (Rayani et al. 2014). The observed variances in the occurrence rate of the disease in various areas could be because of the lifestyles and sample size/population.

By using the *gdh* gene, all of our *G. lamblia* isolates were appropriate for assemblage AII and none of which were found to be part of assemblage B, but amplification of *tpi* gene from other samples exposed the existence of both assemblies A and B, therefore, it seems that *tpi* gene has more power to distinguish between the two major assemblages. These results are consistent with Bertrand et al. described observations (Bertrand et al. 2005).

The majority (78%) of *G. lamblia* isolated in Hamadan belonged to assemblage A genotype. Our results were similar to distributions found in Isfahan (Pestehchian et al.

2012), Tehran (Babaei et al. 2008) and Fars provinces (Sarkari et al. 2012). In contrast, studies in the regions of Egypt (Foronda et al. 2008), Australia (Read et al. 2002), India (Traub et al. 2004) and Kuala Lumpur (Mahdy et al. 2009) reported that distribution of the assemblage B was higher than assemblage A. On the other hand, various studies have reported equal distribution for A and B (Al-Mohammed 2011; Fallah et al. 2008; Nahavandi et al. 2011; Rafiei et al. 2013).

We found that being symptomatic or asymptomatic was independent of the assemblage type, which is consistent with the studies in Kurdistan (Bahrami et al. 2017), Southern Iran (Sarkari et al. 2012), Ahvaz (Rafiei et al. 2013), and Northeast China (Liu et al. 2012). However, some studies reported that assemblages were closely associated with clinical symptoms (Al-Mohammed, 2011; Helmy et al. 2009; Mahdy et al. 2009; Pestehchian et al. 2012). Regarding the research piloted with Read et al. (Read et al. 2002), assemblage B infections were better likely to be asymptomatic. But, a study from Saudi Arabia (Al-Mohammed 2011) reported that clinical symptoms were strongly related with assemblage B. From another point of view, investigations conducted in Isfahan (Pestehchian et al. 2012), Bangladesh (Haque et al. 2005) and Egypt (Helmy et al. 2009) showed that assemblage A was considerably associated with symptomatic patients. The present study demonstrated that sub-assemblage AII was more recurrently connected with urban living and also presenting in the patients with no contact with domestic animals. In the same manner, in a research piloted by Mahdy et al. in Kuala Lumpur (Mahdy et al. 2009), assemblage B was found to be the most usual assemblage which was associated with clinical signs of gastroenteritis (diarrhoea, vomiting, abdominal discomfort, mild fever or nausea). Moreover, they reported that assemblage B was significantly more found in purchasers of unprocessed water, females and children under 12 years of age (Mahdy et al. 2009).

As it was said, *gdh* gene more reliable to determinate the sub assemblage and evaluate the aspects of transmission and the use of the *tpi* gene more suitable to evaluate the source of contamination. Despite the zoonotic potential of *G. lamblia* (World Health Organization 1979), and although outcomes of current molecular epidemiological investigations powerfully propose that zoonotic transmission shows a crucial function in giardiasis epidemiology (Sprong et al. 2009), (as assemblages A and B are known to contaminate individuals and an extensive range of animals), the role of specific animals in the epidemiology of human infections is nevertheless uncertain (Sprong et al. 2009). The sequences of our isolates had 95–100% homology with those of cat (AB569385), sheep (KY083429), and wastewater (KT235915) (Fig. 1). Phylogenetic analysis shows that our isolates were in same cluster with the isolates related to cat, sheep, humans (center and southeastern Iran) and wastewater and in different cluster with isolate related to assemblage D obtained from dog and cat. Therefore by using *gdh* gene, it could be concluded that the route of *Giardia* transmission in Hamadan is likely to be zoonotic. The predominance of zoonotic giardiasis in humans in Hamadan is consistent with studies from Kurdistan (Bahrami et al. 2017), European countries (Sprong et al. 2009), Australia (Palmer et al. 2008) and Northeast India (Traub et al. 2004). Also By using *tpi* gene it has been suggested that people can be infected with *Giardia* of zoonotic origin without direct contact with domestic animals.

Conclusion

According to the present findings, sub-assemblage AII showed a higher prevalence in Hamadan province. On the basis of the bioinformatics explorations it could be resolved that the route of *Giardia* transmission in Hamadan is likely to be zoonotic. Therefore, the necessity of observing the health principles regarding contact with livestock and pet animals is increasingly felt. In this regard, our finding can be utilized as a foundation to detect and mark groups with a bigger chance of giardiasis to develop health principles and preventing programs.

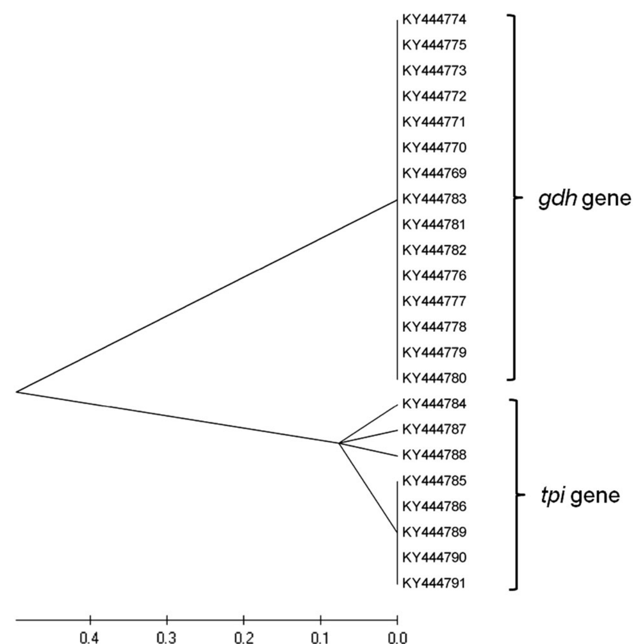

Fig. 2 Phylogenetic relationship among all the twenty three *G. lamblia* isolates sequenced based on both loci in this study

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Authors' contribution M. Kashinahanji and A.H. Maghsood planned the research, carried it out, analyzed data and, assessed clinical records. They also cooperated in the manuscript writing. M. Fallah, M. Saidijam and M. Matini statistical analysis, assisted for sample collection and study design. F. Bahrami and A. Haghighi executed the laboratory assays and cooperated in the manuscript writing and revision.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate The present research was accomplished in agreement with the ethical standards of the Declaration of Helsinki and has been permitted by Hamadan University of Medical Sciences ethics committee (Grant No. 9208072464) with respect to the human rights and a written informed consensus was attained from every participant before gathering data.

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