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Original Article

Sequence and Phylogenetic Analysis of Wild Type Rubella virus isolated in Iran

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Abstract

Background and Aims: Rubella virus is a human pathogen that causes congenital rubella syndrome (CRS) when infection occurs during early pregnancy. Vaccination programs have been remarkably successful in controlling natural rubella infection and CRS. Moreover, ongoing surveillance for all cases of rubella and CRS is a vital component of a prevention program. Although the WHO recommends the use of molecular epidemiology, little is known about circulating strains and genotypes of rubella virus (RUBV) in Iran. This study was designed to analyze the genetic characteristics of the wild type isolated in Iran.

Methods: The partial E1 gene was amplified from the isolated Iran MF rubella virus and Takahashi vaccine strain in comparison with 22 reference strains. The sequence of the E1 gene of the rubella virus isolate was compared by phylogenic analysis.

Results: Nucleic acid sequencing confirmed the isolated virus was Rubella (96 % identity in 784 bases) the sequence was subsequently submitted and registered to the GenBank with accession number DQ975202. The created phylogenetic tree of rubella virus reference sequences showed that the isolated MF rubella virus was classified into genotype 2B.

Conclusion: Based on our data, this is the first report of rubella virus genotyping in Iran. The history of some eradicated viral diseases shows that us how molecular tools are helpful in surveillance. However, more comprehensive molecular epidemiologic studies are required in order to reach Rubella virus elimination goal.

Keywords: Rubella Virus; Genotyping; Phylogenetic analysis

Introduction

Rubella virus as the only member of the Rubivirus genus of the Togavirus family is a human pathogen that causes congenital rubella syndrome (CRS) when infection occurs during early pregnancy, but the infection is generally mild disease in children (1).

The rubella virus genome is ~10,000 nucleotides and encodes five protein products, including three virion proteins: the C or capsid protein and two envelope glycoproteins, E1 and E2. The E1 gene sequence has been used for genotyping and phylogenetic analysis of rubella virus isolates (2-6). To date Rubella viruses from Europe, Asia, and North America have been shown for the most part to group in a single genotype (Rubella Genotype I "RGI") that has a maximum diversity at the nucleotide level of ~5%. However, a limited number of

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viruses from Asia, Europe and Africa, formed a distant phylogenetic branch, differing from RGI viruses by 8% to 10%, which was designated Rubella Genotype II (RGII) (4, 5, 7, 8). These two genotypes (RGI and RGII) belong to the single rubella virus serotype (7).

There is no genotype or sequence information available from the Middle East including Iran and Iran's neighbors except Turkey which has reported the provision g1 genotype for the RV circulating during 2001 to 2003 (9).

Vaccination programs have been remarkably successful in controlling natural rubella infection and CRS. Moreover, ongoing surveillance for all cases of rubella and CRS is a vital component of a prevention program (10, 11). As part of the surveillance component of these efforts, an understanding of the worldwide molecular epidemiology of rubella virus, which is limited, is necessary. Molecular epidemiologic surveillance provides important information on: origin of the virus, virus strains circulating in the country, and, whether these strains have become endemic in the country (2, 4, 5, 9, 12, 13).

After mass vaccination for rubella infection in Iran, seroepidemiological studies have been performed (11, 14, 15), but there is no molecular data for specific characterization of virus particularly virus genotyping up to now (Fig. 1).

This study was designed to analyze the isolated Iranian wild rubella virus by WHO recommended method for describing the genetic characteristics of wild-type rubella viruses.

Methods

Sample collection and virus isolation

In obtaining specimens for rubella molecular genotyping, throat swabs were collected from an unvaccinated seven year old boy who suspected to rubella infection within 4 days of rash onset in Karaj city of Tehran province of Iran. Samples have sent to the cell culture laboratory of medical viral vaccine research and production department in Razi institute for virus isolation. Rubella virus infection was verified by cell culture and specific serological tests; therefore isolated virus was subjected for molecular typing as recommended by WHO (2).

Rubella Virus Vaccine strain

The vaccine virus strain; (Takahashi) was obtained from medical viral vaccine research and production department in Razi institute. Takahashi strain is the only seed strain used for vaccine production in Iran.

Virus Cell Culture

Vero cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 1% triptose phosphate, 2% bovine albumin. Then cell monolayers were inoculated with a suitable virus strain then observed for cytological changes that indicated virus growth.

Preparation of RNA

Vero cell monolayers in 75 Cm^2 T-Flask were inoculated with Rubella virus (vaccine strain and wild isolate). The culture media were ready for virion RNA extraction after five days. Total RNA was extracted from cell culture by using Trizol^{TM} reagent (Invitrogen Life technologies, Karlsruhe) according to the manufacturer's instruction. They were divided to 1.5 ml tube and stored at -70ºC.

cDNA synthesis

First-strand cDNA synthesis was carried out at 42°C for an hour in a 20µl reaction mixture volume, containing viral RNA, 160 pM of the Oligo dT_{18} primer (MBI Fermentas), 10 U/µl of reverse transcriptase, M-MuLV (Revert Aid M-MuLV, Fermantas life sciences) and 0.5 U/µl of RNase inhibitor (Fermentas) in RT buffer.

Polymerase Chain Reaction

By aligning the E1 coding gene sequences from Rubella virus reference strains, a consensus sequence was provided for primer designing. A pair of specific primers (RB1 and RB2) was proposed to cover a recommended E1 gene 739nt window. Designed primers were ordered to be synthesized by CinnaGen (Tehran, Iran). Amplification of target single-stranded cDNA was carried out in a 20µl reaction volume, including 1 µl of each primer (10 pM of each), 0.5 unit of Taq DNA polymerase (CinnaGen, Iran), 2 µl of 10XPCR buffer, 1.5 mM $MgCl_2$, 0.4 µl of 10mM

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nucleotide mix, 2 µl of synthesized cDNA and reached volume with deionized distilled water to a final volume of 20 µl. The sequences of E1 specific primers were as following: RB1 as a sense primer "5' TCCAGCACCCTCACAAGAC3'" and RB2 as an antisense primer "5'CAGTGGTGT-GTGTGCCATAC3'". The PCR cycle parameters were 95ºC for 10S, 60ºC for 30S and 72ºC for 45S repeated for 35 cycles with a hot start at 95ºC for 3 minutes. The final extension step was 5 minutes at 72ºC using PCR system thermal cycler (Techne, Germany). The RB1, RB2 primers produced a fragment of 849bp length. The first negative control included all components of PCR reaction mixture excluding viral RNA but the second negative control had reaction mixtures with uninfected vero cell RNA. PCR products were analyzed by gel agarose electrophoresis containing 1µg/ml ethidium-bromide. Rubella virus specific band of 849bp was identified by fluorescence emerged by using transi-lluminator UV light.

Restriction analysis

To confirm the specificity of the PCR, restriction map of expected PCR product was provided by computational analysis. After selecting a restriction enzyme, an aliquot of the amplicon (10µl) was digested with BamH1 (Fermentas, Germany) restriction enzyme which cut once within the produced fragment. Enzymatic digestion was performed for an hour at 37ºC in a reaction volume of 20µl containing 10µl of PCR sample, 2µl of 10x restriction enzyme buffer and 10U of BamHI restriction enzyme. Digestion products were resolved by agarose gel electrophoresis and visualized by UV illuminator.

Sequence determination

Two PCR products including one Iranian Rubella virus isolate and the vaccine strain (Takahashi), were excised from the gel after electrophoresis, purified by using DNA Extraction kit (Roche, Germany) and were sent for nucleotide bidirectional sequencing.

Computer analysis of nucleotide sequences

Nucleotide sequences were aligned with CLUSTAL W together with reference sequences from the GenBank database. DNADist neighbor phylogenetic tree interface program was used to compute distance matrix from nucleotide sequences and tree construction was performed by the BioEdit software package (Version 7.0.5.3.). The nucleotide sequence of Iranian wild type rubella virus E1 gene was deposited in GenBank under accession number DQ975202.

Results

Sequence analysis of nucleotides and amino acids of the E1 gene of RV

The rubella E1 gene was partially amplified and sequenced (nucleotide position no. 8693 to 9541). The sequence window used in the present study (739 nucleotides) has been recommended by WHO for phylogenetic comparisons of the sequences of a portion of the E1 coding region (2). We analyzed the homology of the nucleotide sequences of the rubella E1 gene, which is amplified from one specimen (Iran "MF" wild RV isolate), and 22 reference strains (Table 1) as well as Takahashi vaccine strain as rubella virus control positive. The E1 gene sequences for Iran wild RV isolate and 22 reference strains and Takahashi vaccine strain were aligned and analyzed. Nucleotide homologies between the obtained DNA fragment from specimen and the reference strains as well as Takahashi vaccine strain are shown in table 2. The identities of E1 nucleotide sequences between reference rubella viruses, Iranian isolate (MF) and Takahashi vaccine strain were between 88.35% and 99.59%. The identity of the nucleotide sequence between the specimen "Iran wild (MF) RV isolate "and the vaccine strain (Takahashi) was 92.09% (Table 2).

Phylogenetic analysis of the E1 gene of rubella virus

A phylogenetic tree was constructed for the nucleotide sequences of the E1 gene from 22 reference strains and Iran wild type (MF) rubella virus isolate by using the neighbor joining (NJ) method (Fig. 2).

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Table 1. Reference Rubella virus strains* and Iran wild (MF) isolate used for phylogenetic analysis (2, 19).

Current name**	GenBank accession no.	Country and year of isolation	Genotype	Comments
To-336 WT JP 67##	AB047330	Japan, 1967		Progenitor wild type RV for Japanese attenuated vaccine
Cendehill BEL 63#	AF188704	Belgium, 1963	1A	Attenuated Vaccine
HPV77 US 61#	M30776	USA, 1961		Attenuated Vaccine
I-9 IS 75	AY968207	Israel 1975		
I-13 IS 79	AY968208	Israel 1979	1B	
I-34 IS 88	AY968209	Israel 1988		
QUI ELS 02	AY968211	El Salvador 2002	1C	
BUR US 91	AY968212	USA 1991		
P-13 PAN 99	AY968217	Panama 1999		
SAL-CA US 97	AY968206	USA 1997		
NC JP 90	AY968214	Japan 1990		
SAI-1 JP 94	AY968216	Japan 1994	1D	
T14 CH 02	AY968210	China 2002		
M-1 MAL 01	AY968221	Malaysia 2001	1E	
TS10 CH 00	AY968213	China 2000		
TS38 CH 00	AY968215	China 2000	1F	
BRD1 CH 79	AY258322	China 1979		
BRD2 CH 80#	AY258323	China 1980	2A	Attenuated vaccine
TS34 CH 00	AY968318	China 2000		
I-11 IS 68	AY968219	Israel 1968		
TAN IND 00	AY968220	USA 2000	**2B**	
IRAN RV 99	**DQ975202**	**Iran 1999**		Iran wild RV isolated in 1999
RA27/3 US 64#	L78917	USA 1964		Attenuated vaccine

* Reference viruses for provisional genotypes 1g and 2c are not available yet.
** Strains are named using old or previously published identifiers.
# Attenuated vaccine virus for which original wild-type virus has been lost

The constructed phylogenetic tree revealed two major clusters of RGI and RGII. In this study, the strains that belonged to clade 1 were subdivided into 6 minor genotypes (1A to 1F). Takahashi, the vaccine strain was classified into clade 1, genotype 1A. The diversity among the genotypes in clade 1 (RGI) ranged from 1.65% to 7.57% (average 4.61%). The diversity among the genotypes in clade 2 (RGII) ranged from 2.62% to 8.19% (average 5.41%). The difference between the RG I and RG II viruses in the worldwide tree was 7.00%-11.65% (average 9.32%). The constructed tree was clearly demonstrated the position of "Iran (MF) rubella virus 1999" isolate into genotype 2B. The difference between Iran isolate and RGI viruses ranged from 7.70% to 11.39% (average 9.05%), but the diversity of the Iran isolate and RGII viruses varied from 4.20% to 7.89% (average 6.05%), but the difference was at least between Iran isolate and RGII genotype 2B viruses from 4.20% to 4.49% (average 4.35%). The homology of the Iran MF isolate and two RV vaccine strains, Takahashi and RA27/3 were 92.09% and 91.03% respectively (Table 2).

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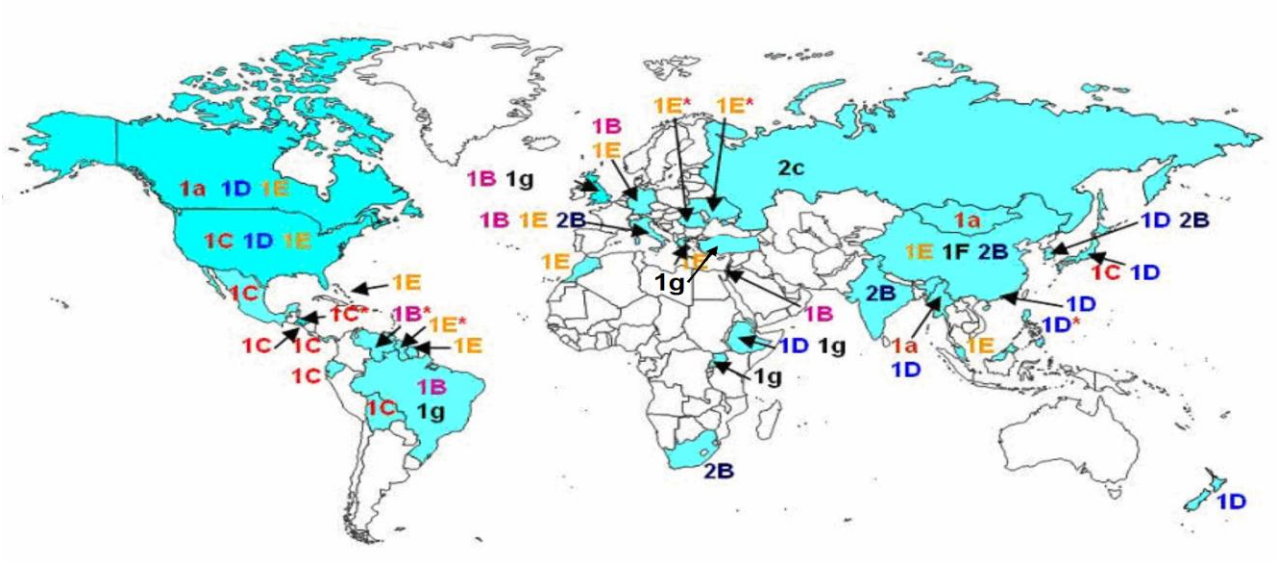


Fig. 1. Genotype distribution of Rubella viruses 1985-2007. All genotype information reported and published information until 2007 are shown on the map. Countries from which no genotype information is available are white including Iran. Any genotype with an asterisk indicates that the information was not obtained from the indicated country, but rather from a country into which the virus was imported. Since the time period shown is 20 years, virus circulating in any particular country may currently be different. However, in countries without strong rubella control programs, the indicate genotypes are likely still circulating.

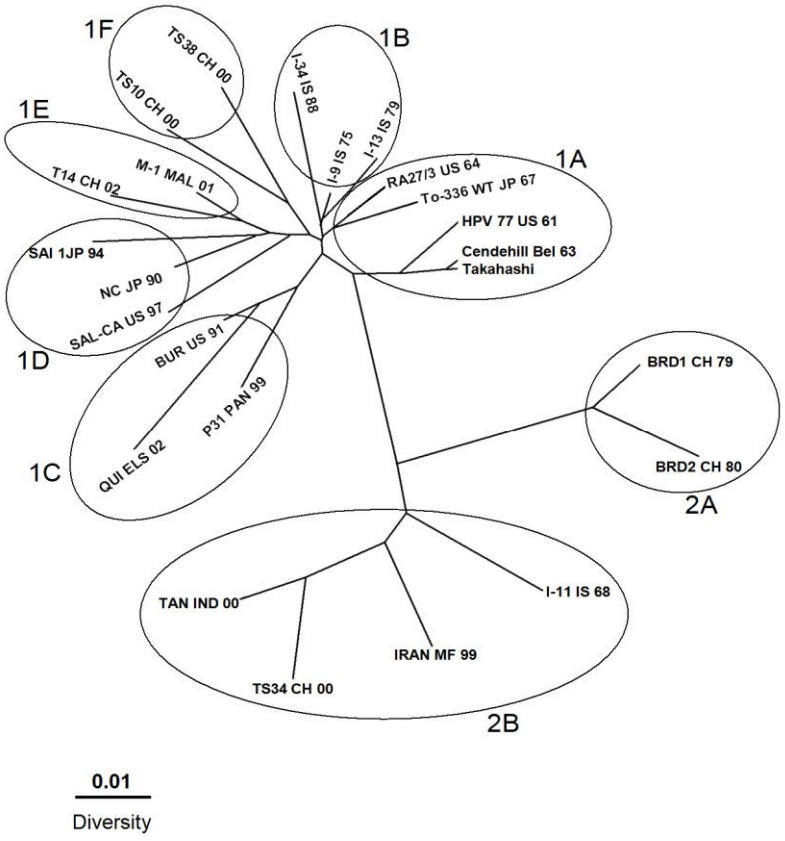


Fig. 2. Phylogenetic tree of rubella virus references sequences, listed in table 1, by using 739 nucleotides; 8731-9469. The phylogenetic tree was constructed by using Neighbor joining method. Unrooted tree was made by the DNADIST version 3.5c, program to compute distance matrix from nucleotide sequences. There are no provisional genotypes 1g and 2c in the constructed tree.

Discussion

In this study we have shown the phylogenetic analysis of the Iranian rubella virus isolate (MF) based on WHO guideline (2). Today, with the great achievements in fields of serology, cell culture, virus

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Table 2. Sequence Identity Matrix of E1 nucleotide sequences between 22 references RV, Iran wild RV isolate and Takahashi RV vaccine strain. The identity matrix shows the proportion of identical residues between all of the sequences in the alignment as they were previously aligned by "Clustal W multiple alignment" in BioEdit Sequence Alignment Editor Program.

		Nucleotides (739 nt)											
Genotype		1A			1B			1C			1D		
GenBank acc#		AB047330	AF188704	M30776	AY968207	AY968208	AY968209	AY968211	AY968212	AY968217	AY968206	AY968214	AY968216
1A	AB047330	ID	96.95	96.54	97.93	96.81	96.26	93.93	96.81	96.09	95.97	96.96	95.23
	AF188704	96.95	ID	98.09	97.37	96.25	95.40	93.64	95.81	94.94	95.11	95.68	94.66
	M30776	96.54	98.09	ID	96.67	96.11	94.69	93.79	95.67	94.80	94.97	95.26	94.23
1B	AY968207	97.93	97.37	96.67	ID	98.35	97.24	94.79	97.51	96.65	96.53	97.23	95.94
	AY968208	96.81	96.25	96.11	98.35	ID	96.54	94.21	96.52	95.52	96.25	96.39	95.37
	AY968209	96.26	95.40	94.69	97.24	96.54	ID	93.36	96.25	94.66	95.12	94.97	94.38
1C	AY968211	93.93	93.64	93.79	94.79	94.21	93.36	ID	96.54	94.66	93.20	93.20	92.43
	AY968212	96.81	95.81	95.67	97.51	96.52	96.25	96.54	ID	97.09	95.67	96.24	94.93
	AY968217	96.09	94.94	94.80	96.65	95.52	94.66	94.66	97.09	ID	94.80	95.82	94.79
1D	AY968206	95.97	95.11	94.97	96.54	96.25	95.12	93.20	95.67	94.80	ID	96.55	95.25
	AY968214	96.96	95.68	95.26	97.23	96.39	94.97	93.20	96.24	95.82	96.55	ID	96.54
	AY968216	95.23	94.66	94.23	95.94	95.37	94.38	92.43	94.93	94.79	95.25	96.54	ID
1E	AY968210	95.53	94.95	94.24	96.52	95.67	94.82	92.59	94.94	94.79	95.26	95.97	95.68
	AY968221	96.52	95.95	95.24	97.51	96.67	95.53	93.76	95.66	95.80	96.25	97.52	96.38
1F	AY968213	95.56	94.41	93.99	96.55	95.42	95.43	92.65	95.12	93.95	95.42	95.28	94.83
	AY968215	96.12	95.41	95.56	96.25	95.69	94.27	93.21	95.11	94.52	94.98	94.83	93.96
2A	AY258322	90.53	90.90	91.82	91.13	90.68	89.32	89.73	90.82	90.81	90.11	90.57	90.69
	AY258323	89.51	90.19	91.11	90.41	89.96	89.53	89.01	90.10	89.60	88.60	89.38	89.49
2B	AY968318	90.39	91.07	91.53	90.68	90.38	88.86	89.12	89.90	89.72	89.77	89.78	88.80
	AY968219	91.36	92.01	92.32	92.25	93.00	90.77	89.95	91.33	90.54	91.03	90.44	90.10
	AY968220	90.56	91.23	91.70	91.46	91.77	89.66	89.61	91.00	90.20	90.26	90.87	90.22
	Iran RV 1999 (DQ975202)	91.67	91.84	92.30	91.76	92.08	90.28	89.61	91.61	90.20	90.72	90.56	90.22
	Takahashi	96.93	99.59	97.94	97.35	96.22	95.37	93.89	96.07	95.19	95.07	95.65	94.62
	RA 27/3	97.80	96.96	96.54	98.21	97.24	96.54	94.23	96.95	96.09	96.11	96.82	95.81

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Table 3. Table 2 Continued.

Nucleoids (739 nt)											
1E		1F		2A		2B				Vaccine strain	Vaccine strain
AY968210	AY968221	AY968213	AY968215	AY258322	AY258323	Takahashi	AY968219	AY968220	Iran RV 1999 (DQ975202)	Takahashi	RV 27/3
95.53	96.52	95.56	96.12	90.53	89.51	96.93	91.36	90.56	91.67	96.93	97.80
94.95	95.95	94.41	95.41	90.90	90.19	99.59	92.01	91.23	91.84	99.59	96.96
94.24	95.24	93.99	95.56	91.82	91.11	97.94	92.32	91.70	92.30	97.94	96.54
96.52	97.51	96.55	96.25	91.13	90.41	97.35	92.25	91.46	91.76	97.35	98.21
95.67	96.67	95.42	95.69	90.68	89.96	96.22	93.00	91.77	92.08	96.22	97.24
94.82	95.53	95.43	94.27	89.32	89.53	95.37	90.77	89.66	90.28	95.37	96.54
92.59	93.76	92.65	93.21	89.73	89.01	93.89	89.95	89.61	89.61	93.89	94.23
94.94	95.66	95.12	95.11	90.82	90.10	96.07	91.33	91.00	91.61	96.07	96.95
94.79	95.80	93.95	94.52	90.81	89.60	95.19	90.54	90.20	90.20	95.19	96.09
95.26	96.25	95.42	94.98	90.11	88.60	95.07	91.21	90.26	90.72	95.07	96.11
95.97	97.52	95.28	94.83	90.57	89.38	95.65	90.44	90.87	90.56	95.65	96.82
95.68	96.38	94.83	93.96	90.69	89.49	94.62	90.10	90.22	90.22	94.62	95.81
ID	97.38	94.55	94.39	89.63	88.74	94.92	89.97	89.15	89.93	94.92	96.11
97.38	ID	96.12	95.68	90.69	89.81	95.92	91.03	90.53	91.00	95.92	96.81
94.55	96.12	ID	95.86	89.05	88.64	94.67	89.58	89.39	90.92	94.67	95.57
94.39	95.68	95.86	ID	90.11	89.09	95.66	90.63	90.13	91.36	95.66	96.12
89.63	90.69	89.05	90.11	ID	97.38	91.14	92.86	93.13	92.22	91.14	90.54
88.74	89.81	88.64	89.09	97.38	ID	90.58	92.90	92.87	92.11	90.58	89.68
88.35	89.74	88.90	89.65	92.37	91.81	91.00	94.51	97.37	95.51	91.00	89.94
89.97	91.03	89.58	90.63	92.86	92.90	91.96	ID	95.53	95.09	91.96	91.52
89.15	90.53	89.39	90.13	93.13	92.87	91.48	95.53	ID	95.80	91.48	90.73
89.93	91.00	90.92	91.36	92.22	92.11	92.09	95.09	95.80	ID	92.09	91.03
94.92	95.92	94.67	95.66	91.14	90.58	ID	91.96	91.48	92.09	ID	96.94
96.11	96.81	95.57	96.12	90.54	89.68	96.94	91.52	90.73	91.03	96.94	ID

isolation, molecular techniques, alternative sampling methods and data management which had brought us new aspects for precise disease control, prevention, monitoring and surveillance (9, 16-18).

Recently, some specific advices are recommended by World Health Organization in order to rubella immunization and surveillance programs. Sequence (genotype) information is providing a valuable data to epidemiological information in determining

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whether outbreaks are due to imported or indigenous rubella virus strains and can be helpful in evaluating vaccination campaigns.

Seroepidemiological survey conducted in Iran after mass vaccination in 2003 but there was no sequencing data of rubella virus in this country. In this study, we provide the first evidence of rubella genotype in Iran.

Viruses in clade 2 (Rubella Genotype II "RGII") have been found only in the eastern hemisphere (Asia, Europe, and Africa). Genotype 2A was isolated only in China in 1979 and 1980 and has not reappeared since then. Genotype 2B is distributed more widely than other genotypes in clade 2. Genotype 2c has been found only in Russia (12).

The eradication efforts of measles and rubella in Iran are in the way. Although the vaccination program brought the appropriate immune coverage among the vaccines (11, 14, 15), but the immunity status is not clear in different region of Iran. Moreover, we do not know what and how many rubella genotype(s) are currently circulating in our country. Molecular phylogenetic analysis is useful tool for rubella virus surveillance and in order to reach to this goal, obtaining sequences from circulating virus is necessary. Nevertheless, no data was reported from RV genotypes from our neighboring countries except Turkey (9). However, RV genotype 2B was reported from India, China, South Korea in Asia, and from South Africa and England.

In this study, phylogenetic analysis and alignment of nucleotide sequences of the rubella virus E1 gene belonging to the reference strains and Iranian viruses wild type (MF) and Takahasi vaccine strain has been performed. The phylogenetic analysis showed that the Iran wild "MF" isolate was classified into genotype 2B (RG II) which is mostly introduced from Asia but is more distributed in the world in comparison with the other genotypes in clade 2 (2, 4, 12).

The Iran MF wild RV was isolated in 1999. It is notable that two RV reference strains of three reference viruses in genotype 2B were isolated in 2000 in India and China (TS34 CH 00 and TAN IND 00), thus the isolation of 2B genotype between 1999 and 2000 shows the viruses were coincident and may have been circulating during this time period.

However, the rubella virus has been isolated from an Iranian boy with rubella infection symptoms with no history of rubella vaccination- in mass vaccination- program in Iran, therefore it might be the possibility of virus invasion through the east borders, since the same rubella virus genotype (2B) have been reported in India and China in 2000.

Limited number of specimen and the virus isolation from suspected cases or even direct RT-PCR sequencing makes a gap for molecular epidemiology studies. No more isolate makes a limitation to draw conclusions about the circulation genotypes in Iran. Therefore widespread organization is needed for specimen gathering to asses all suspicious cases and also to complete the rubella virus genotypic picture.

In conclusion, this was the first time that genotype of Iran wild rubella virus isolate was introduced based on recommended WHO standard protocol for molecular genotyping. Molecular epidemiological study need to have data from circulating viruses as our country is undertaking measles and rubella elimination interference between viruses belonging to the same family or between members of unrelated ones upon coinfection of cells has been reported (18, 19).

Generally, Infection of a cell with two viruses could result in growth and maturation of both viruses, which might be beneficial to one of the viruses such as coinfection by adenovirus, and adeno-associated viruses. Coinfection could also end up in growth inhibition of one of the viruses by the other such as infection of cells with enteroviruses, which would cause growth inhibition of poliovirus (10, 11). This kind of interference has significant application value in certain systems. An example of such interference is the effect of enterovirus infection with live - attenuated poliovirus vaccine which slows down replication of vaccine virus inside the cells, resulting inefficiency of vaccine - induced protection (10, 11). The other example is the effect of reovirus on environmental detection of enteroviruses (17).

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In this study we wanted to test the interference between reovirus and poliovirus, which can coexist in alimentary tract. We used Vero cells for coinfection experiments, which support growth of both viruses.

The results indicated a marked inhibition in poliovirus production in cells that were infected first with reovirus and then reinfected with poliovirus.

When the harvest of coinfected cells were used to infect another set of cell monolayer (second passage), only reovirus was produced. Electron microscopy of the infected cell lysate revealed that only reovirus particles were present. This inhibitory effect was also observed in sections of poliovirus superinfected cells where only reovirus particles were present inside the cytoplasm and no poliovirus particle was observed. In simultaneous infection of cells with reo and poliovirus it was observed that poliovirus replicated efficiently and resulted in cell lysis. The mechanism of reovirus inhibition of poliovirus growth remains to be determined.

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