

Detection and Molecular Characterization of Avian Rotaviruses in Broiler Farms in Guilan

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Abstract:

BACKGROUND: Avian rotaviruses are one of the important agents of enteritis in birds and have a worldwide distribution. These viruses have an important role in runting and stunting syndrome in chickens.

OBJECTIVES: The aim of this study was detection and molecular characterization of avian rotaviruses in broiler farms in Guilan province.

METHODS: In this study, 100 samples were collected on different farms from the intestines of healthy and diseased broiler chickens and tested by RT-PCR targeting of the NSP4 gene. Sequencing of NSP4 gene has been used for characterization of detected viruses.

RESULTS: Avian rotaviruses were detected in 46% of samples by RT-PCR. Partial sequencing of seven NSP4 genes and phylogenetic analysis indicated that the viruses belonged to group A avian rotaviruses and were 97.52% to 100% homologous. Sequence identity between viruses with previous chicken rotavirus sequences was 31.68% to 99.5%. In comparison with turkey rotavirus Ty-1 and pigeon rotavirus, the homology was 76.98% to 78.22% and 74.75% to 76.98%, respectively. Evident differences were detected between the rotaviruses studied and human and pig rotaviruses with homologies of 31.19% to 38.86% for pig and 47.77% to 48.02% for human viruses.

CONCLUSIONS: This study was the first molecular characterization of avian rotaviruses in Iran. Results of this study showed that the group A avian rotaviruses are one of the considerable enteric pathogens in broiler chickens of Guilan province. Further study on the whole genome structure could elucidate the evolutionary process of avian rotaviruses circulating in Iran.

Keywords:

Avian rotaviruses, Broiler chickens, Iran, NSP4 gene, Phylogenetic study

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Introduction

Enteritis has detrimental economic impact on the poultry industry because it decreases feed absorption and retards growth. Rotaviruses are one of the most prevalent causes of viral enteritis and are found in both diseased and healthy birds (Villarreal et al., 2006; Moura-Alvarez et al., 2013; Beserra et al., 2014). Rotavirus infections have a worldwide distribution (Knipe et al., 2013). The first report of avian rotaviruses was from turkey poults suffering from enteritis in the USA in 1977 and in the UK in 1978 (McNulty et al., 1978). These viruses belong to the family Reoviridae and are non-envelope icosahedral particles that have double-stranded RNA with an 11-segmented genome. These segments encode 12 structural and non-structural proteins (VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6).

Group and subgroup-specific antigens are located on the VP6 protein, while those of serotypes are located on VP4 and VP7. The rotaviruses have been classified into eight groups (A-H) based on the VP6 protein (Estes et al., 1989). Groups D, F, and G are bird specific (Trojnar et al., 2010) and group A is common to birds and mammals. NSP4 is a nonstructural protein that acts as an intracellular receptor for immature viral particles and interacts with capsid proteins during morphogenesis of the virus. NSP4 is also an enterotoxin that is capable of causing diarrhea (Kirkwood et al., 1997). Mutation in the NSP4 gene can cause variations in the virulence of avian rotaviruses (Zhang et al., 1998). Because the NSP4 gene is somewhat well-conserved, it can be used to screen for rotaviruses in poultry samples (Pantin-Jackwood et al., 2007; Kirkwood et

al., 1997).

Rotavirus transmission occurs directly and indirectly through the fecal-oral route. Natural infection by rotaviruses occurs in chickens, turkeys, pheasants, partridges, ostriches and ducks (Silva et al., 2012). The disease symptoms usually appear at less than 6 weeks of age (Dhama et al., 1986). Rotavirus infections can be accompanied with other gastrointestinal pathogens (Day et al., 2013; Nunez et al., 2013; Roussan et al., 2012). They are one of the agents involved in runting and stunting syndrome (RSS) in chickens and poult enteritis syndrome (PES) in poults (Nunez et al., 2016; Kang et al., 2012; Otto et al., 2006). RSS is a syndrome with transient diarrhea followed by growth retardation. It has mild clinical symptoms, but causes substantial economic loss and disturbs growth uniformity. Intestinal villous atrophy is frequently detected in chicks examined from flocks with RSS (Otto et al., 2006).

The current study was performed on broiler farms in Guilan province of northern Iran, which is a region of intensive commercial poultry production and because of the significant role of a healthy gastrointestinal tract for optimal weight gain.

Materials and Methods

Sampling: One hundred 1-6 week-old chickens were selected from five broiler farms in five cities in Guilan province (37.2809° N and 49.5924° E). Five farms were randomly selected, each for a specific age range, and 10 healthy and 10 diseased birds were randomly collected from each farm. The sample characteristics are shown in Table 1. The clinical signs of diseased birds were growth retardation, stunting and diarrhea. In some chickens with severe di-

arrhea, the intestinal walls were distended and gaseous and the contents were frothy. A few birds showed no diarrhea, but had growth retardation.

The birds were euthanized and the intestines were removed and 1 cm each of the duodenum, jejunum, and ileum were excised. Next, the intestines were mixed with three parts of sterile PBS and homogenized. The homogenized tissue was vortexed for 2 min and then centrifuged at $2000 \times g$ for 15 min and the supernatants were harvested into 1.5 ml Eppendorf tubes. The microtubes were stored at -20°C for the next steps.

RNA extraction and cDNA synthesis: Viral RNA was extracted from 100 μl of the sample supernatants using an RNX solution (SinaClon) according to manufacturer's instructions. The extracted RNA was stored at -70°C . The generation of cDNA was performed using Fermentas cDNA synthesis kit. Two-step RT-PCR was performed to amplify a distinct region (630 bp) of the nonstructural NSP4 gene. The synthesis of cDNA was carried out according to kit protocol in which 1 μL of random hexamer was added to 5 μl of extracted RNA (master mix 1). After that, the prepared master mix was incubated at 65°C for 15 min and at 5°C for 1 min. In the next step, 7.25 μl distilled water, 4 μl buffer 5X, 0.25 μl RNase inhibitor, 2 μl dNTP (10 mM) and 0.5 μl of RT enzyme were added to the master mix. The total volume (20 μl) was incubated in a thermocycler according to the following program: 25°C for 5 min, 42°C for 60 min, 95°C for 5 min and 5°C for 1 min.

PCR for NSP4 gene amplification: Rotaviruses were detected using a set of primers targeting the well-conserved region of the NSP4 gene. The sequence of the primer was: forward (5'-GTG CGG AAA GAT

GGA GAAC-3') and reverse (5'-GTT GGG GTA CCA GGG ATT AA-3') (Pantin-Jackwood et al., 2007). The amplification was performed in a final volume of 20 μl containing 2 μl distilled water, 13 μl of PCR 2X master mix (SinaClon; Iran), 2 μl of primer (10 μM) and 3 μl of cDNA. Amplification was performed with a 35-cycle thermal profile of 94°C for 2 min, 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec and 72°C for 10 min. The reaction products were visualized under UV light.

Phylogenetic analysis: The RT-PCR products were sequenced by Bioneer (Korea). Multiple sequence alignments were performed with ClustalW and a phylogenetic tree was constructed with MEGA 7 software using the neighbor-joining method with 1000 bootstrap replicates to assign confidence levels to the branches. The ARV sequences were aligned and compared with reference strains (Fig.1). The sequences obtained were submitted to the NCBI GenBank database. The amplified part of the NSP4 gene sequence of the ARV was submitted to GenBank with the following accession numbers: MH106448-MH106454.

Results

Rotaviruses were identified in 46% of the samples. ARVs were detected in 66% of diseased and 26% of healthy birds. The percentage of positive and negative cases on each farm is shown in Fig. 2. The RT-PCR products of seven positive samples were sequenced and compared with previously published sequences for chicken, pigeon, turkey, pig and human rotaviruses.

All rotaviruses sequenced in this study were placed in a common group (group A) and had a sequence homology of 97.52% to 100% (Table 2). In addition, the homol-

Table 1. Characteristics of broiler flocks from RT-PCR for avian rotaviruses in Guilan province in 2017.

Farm location	Age (week)	No of samples	Geographical latitude and longitude
Rasht	1W	20	37°16'51"N 49°34'59"E
Langarud	2W	20	37°11'49"N 50°09'13"E
Talesh	3W	20	37.801424°N 48.906695°E
Sowmesara	4W	20	37°18'42"N 49°19'19"E
Shaft	6W	20	37°10'13"N 49°23'59"E

Table 2. Percentage of identity of partial nucleotide sequences of NSP4 of Iranian AvRVs to those of ARV reference strains.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1 MH106448																				
2 MH106454	99.50																			
3 MH106449	100	99.50																		
4 MH106450	100	99.50	100																	
5 MH106453	100	99.50	100	100																
6 MH106452	98.27	97.77	98.27	98.27	98.27															
7 MH106451	98.02	97.52	98.02	98.02	98.02	99.75														
8 Rota1063-5	97.77	97.28	97.77	97.77	97.77	99.50	99.26													
9 Rota1063-7	97.77	97.28	97.77	97.77	97.77	99.50	99.26	100.00												
10 AB065285	78.22	78.71	78.22	78.22	78.22	77.23	76.98	76.98	76.98											
11 KX826054	76.49	76.98	76.49	76.49	76.49	75.00	74.75	74.75	74.75	91.09										
12 JX4747551	81.44	81.44	81.44	81.44	81.44	81.93	82.18	81.44	81.44	74.75	72.52									
13 K932157	85.15	85.15	85.15	85.15	85.15	84.41	84.65	83.91	83.91	75.00	74.26	85.40								
14 JX474761	82.92	82.92	82.92	82.92	82.92	82.92	83.17	82.43	82.43	74.01	72.28	95.05	86.63							
15 KR052712	38.37	38.37	38.37	38.37	38.37	38.61	38.86	38.37	38.37	38.61	37.87	38.12	38.61	38.12						
16 KF142490	34.41	34.65	34.41	34.41	34.41	33.91	33.66	33.42	33.42	35.64	36.39	35.89	33.66	34.41	30.69					
17 X362567	31.68	31.68	31.68	31.68	31.68	31.44	31.19	31.68	31.68	30.94	31.68	32.43	35.15	31.68	35.64	29.21				
18 KJ752082	32.67	32.43	32.67	32.67	32.67	31.68	31.93	31.44	31.44	34.41	32.18	35.64	33.42	34.65	48.27	32.92	31.44			
19 KT148607	48.02	48.02	48.02	48.02	48.02	47.77	48.02	47.77	47.77	47.28	44.31	48.02	47.77	48.76	34.16	32.18	31.44	31.68		

ogy of the NSP4 gene sequence with other previously reported chicken rotaviruses was 31.68% to 99.5%. Compared to the turkey rotavirus Ty-1 (AB065285) in group A, the homology was 76.98% to 78.22%. The range of similarity of the detected chicken rotaviruses with pigeon rotavirus pigeon-wt/NIE/NIE08_A_568/2008/GXP (KX826054) was 74.75% to 76.98%. There were obvious differences among the nucleotide sequences of the rotaviruses detected in the current study and pig and human viruses. The homology was 31.19% to 38.86% for pig and 47.77% to 48.02% for human viruses.

Discussion

Enteric viruses can cause economic loss

due to enteritis, decreased feed absorption, reduced weight gain, diarrhea, and death (Guy ,1998). Rotaviruses are important causes of enteritis and have a worldwide distribution. These viruses are agents of RSS in chickens and can be found in healthy and diseased birds (Otto et al., 2012). There has been no detailed study on the prevalence and molecular characteristics of rotaviruses in Iran. Because of the negative effects of rotavirus infections on broiler chickens, the current study was performed to determine the rate of avian rotavirus infection in broiler farms in northern Iran.

In the current study, the intestinal segments of 100 broiler chickens were tested. Because the rotavirus can exist in both

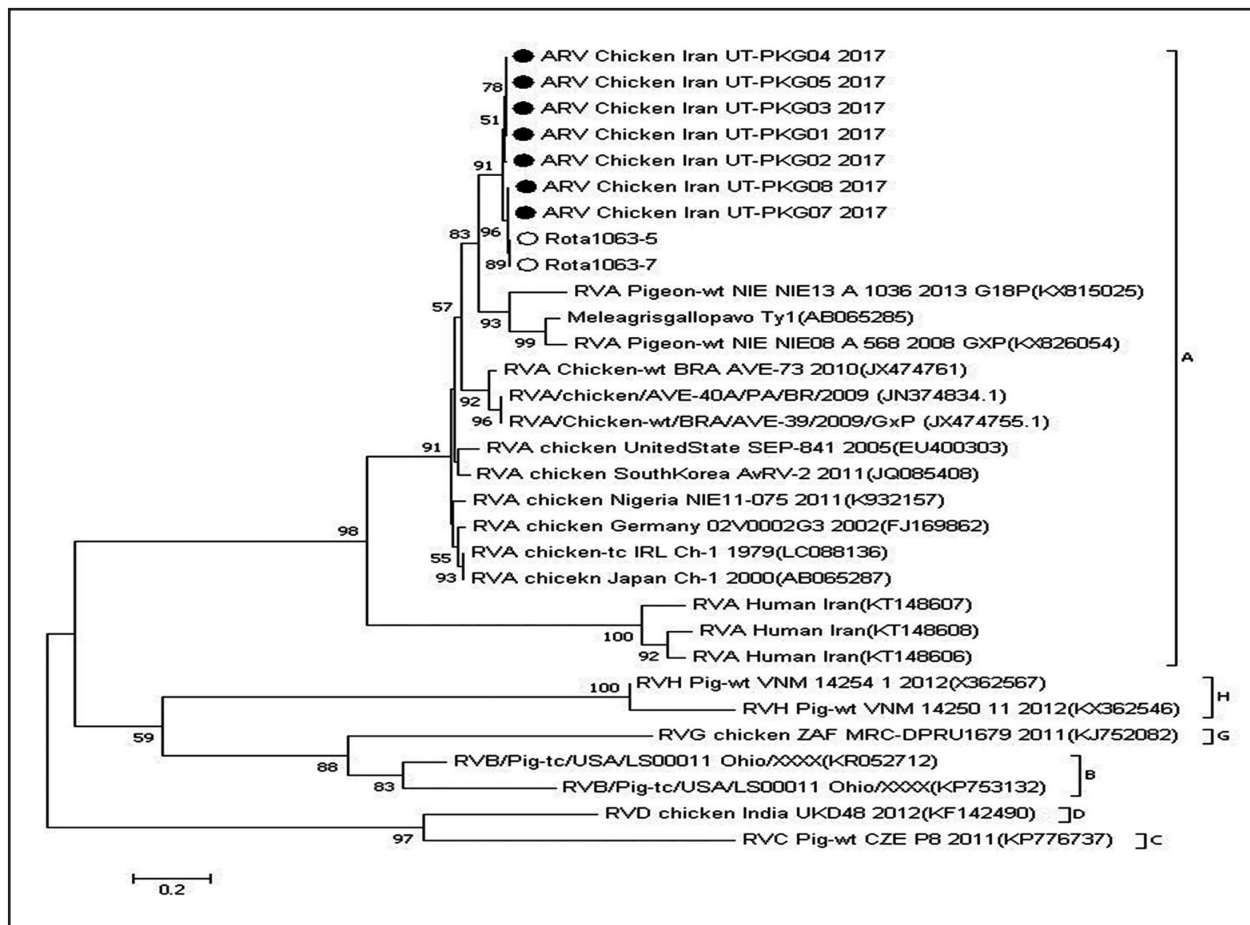


Figure 1. Phylogenetic tree based on partial sequencing of NSP4 gene showing the relationship between Iranian strains from healthy and diseased broiler chickens and other AvRvstrains. Viruses detected in current study are denoted by black circles and two viruses previously identified in Iran are denoted by white circles. The scale bar represents the distance between sequence pairs. The sequences were obtained from GenBank.

healthy and diseased birds, both types were selected for sampling. Rotaviruses were detected in 46% of samples. The rate of infection in diseased birds was higher than in healthy ones. The higher percentage of rotaviruses in diseased birds emphasizes their probable role in the poor performance, growth retardation, enteritis and related symptoms that are attributed to this virus.

All of the sequenced viruses belonged to group A rotaviruses. In a study on detection of rotaviruses in chickens (2006), Villarreal et al. (Villarreal et al., 2006) reported a rotavirus frequency of 48.7% in samples from flocks with diarrhea symptoms, 46.4% in flocks with delayed growth and 30% in

healthy flocks using polyacrylamide gel electrophoresis (PAGE). In another survey from northern Germany (2006), 32 of 34 chicks with RSS were positive for rotavirus and group A rotaviruses were identified by RT-PCR in samples of 20 chicks with RSS. Using the PAGE method, groups A, D, G, and F were identified. Microscopic observation suggested that group D caused more severe villous atrophy in the intestines and played a major role in the pathogenesis of RSS (Otto et al., 2006).

A study for the detection of rotaviruses in commercial chicken and turkey farms in the United States (2006) indicated the presence of rotavirus in 46.5% of broiler chick-

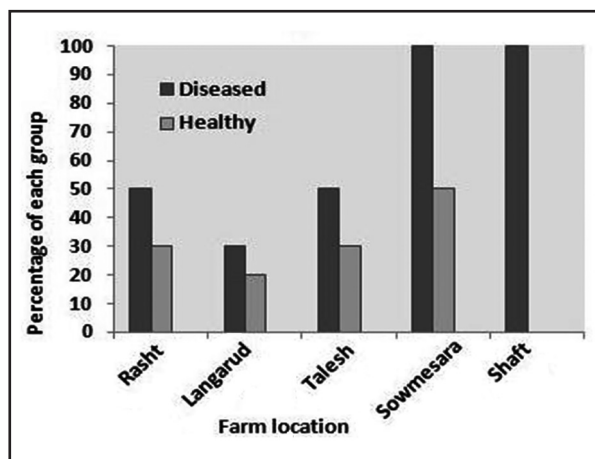


Figure 2. Prevalence of avian rotaviruses in diseased and healthy birds by broiler farms of Iran in 2017.

en farms (Pantin-Jackwood et al., 2008). Group A and D rotaviruses have been detected by the PAGE method from diarrhetic poultry in central India in 2006; however, the prevalence of group D was higher (77.8%) than group A (22.22%) (Savita et al., 2008). Bezerra et al. reported the occurrence of group D rotavirus in apparently healthy chickens in Brazil (2012) (Bezerra et al., 2012). In another study from India (2013), Kattoor et al. detected group D rotavirus in 1-2 week old commercial broiler chicks with symptoms of diarrhea (Kattoor et al., 2013). An epidemiological study for molecular detection of groups A and D rotaviruses in healthy and diseased birds in Nigeria (2017) done by Pauly et al. detected groups A and D in 51.9% and 48.1% of healthy and 18.9% and 29.7% in diseased chickens, respectively (Pauly et al., 2017).

Otto et al., detected rotaviruses in 46.2% of samples taken from chickens and turkeys in European and Bangladeshi farms between 2005 and 2008. Groups A, D, F and G were recognized. Groups D and A were the most abundant and F and G were the least. Groups F and G were shown in the PAGE pattern in only 2% of samples. Following

the first study, they detected rotaviruses in 85% of samples and group A and D were detected by real time RT-PCR in high percentages again between 2009 and 2010 (Otto et al., 2012). This study emphasizes the high prevalence of groups D and A in diseased birds. In a molecular survey for detection of enteric viruses in commercial broiler and layer chickens with a history of enteritis in Korea done by Koo et al. (2013), 85.3% of investigated farms were positive for enteric viruses, but rotaviruses were detected only in 5.9% of samples in the RT-PCR. The two viruses sequenced in their study belonged to group A rotaviruses (Koo et al., 2013).

Studies have shown that groups A and D are predominant groups among poultry in both diseased and healthy birds. Phylogenetic analysis of the NSP4 gene detected in the current study indicated a sequence homology of 97.52% to 100%. Pantin-Jackwood et al. reported a sequence identity between rotaviruses of chicken origin from the US of 89.2% to 100% (Pantin-Jackwood et al., 2008). These findings indicate that chicken rotaviruses of adjacent areas have high sequence homology. In the present study, the homology of the NSP4 gene sequence with other chicken rotaviruses showed a wide range of 31.68% to 99.5%. The lowest homology was for RVG/chicken/ZAF/MRC-DPRU1679/2011 (KJ752082) and RVD/chicken/India/UKD48/2012 (KF142490). These two viruses belonged to groups G and D, which may explain the reason for the low identity with Iranian viruses. Turkey rotavirus Ty-1 and pigeon rotavirus pigeon-wt/NIE/NIE08_A_568/2008/GXP have been placed in a separate clade from chicken AvRVs. This separation could be due to a homology of 76.98% to 78.22% and 74.75% to 76.98% between Iranian

chicken viruses and turkey and pigeon viruses, respectively.

The assortment of turkey and pigeon rotaviruses in a separate group from that of chicken-based viruses on the NSP4 gene could be a barrier for interspecies transmission (Jindal et al., 2010). In a study by Mori et al. (2002) a group A chicken AvRV (Ch-1) NSP4 gene was sequenced and Ch-1 showed only 78.79% amino acid identity with other avian rotaviruses. Also, the identity between strain Ch-2G3 and pigeon PO-13 was 79.3% (Mori et al., 2002). Another study comparing turkey AvRVs and chicken Ch-1 strain (2007) showed 76.3% and 76.9% identity (Pantin-Jackwood et al., 2007). Turkey rotaviruses detected by Jindal et al. (2010) of PES showed a sequence homology of 66% to 69% with previously published chicken AvRVs. Phylogenetic analysis of the NSP4 gene sequence placed turkey rotaviruses in a separate clade from chicken rotaviruses (Jindal et al., 2010).

NSP4 gene nucleotide sequences of chicken AvRVs and mammal rotaviruses showed very low identity percentages of 31.19% to 38.86% and 47.77% to 48.02% for pig and human viruses, respectively. Although human RVA (KT148607) falls into group A, its homology with chicken AvRVs is low. This difference may indicate that the human group A rotaviruses have separated from a common ancestor in the evolutionary process (Trojnar et al., 2009; Mori et al., 2002). Mori et al. (2002) showed that amino acid sequences deduced from the NSP4 genes of group A avian rotaviruses have low identities with mammalian strains (31% to 37%) (Mori et al., 2002). The findings of Trojnar et al. (2009) confirm the wide distance between avian and mammalian rotaviruses, for which the identity of chicken AvRV to

mammalian RV NSP4 sequences was low (30.8% to 33.7%) (Trojnar et al., 2009).

Results of this study showed that the group A avian rotaviruses are one of the considerable enteric viruses in broiler chickens of Guilan province. The high percentage of AvRVs in the diseased birds in this study indicates that this virus has contributed to the symptoms stated previously. Group A mammalian rotaviruses are distant to avian ones in the phylogenetic tree and probably have separated in the evolutionary process. The results of this study agree with those of previous works done. Further study is needed for molecular characterization of additional viruses and sequencing of genes other than NSP4. Molecular study of avian rotaviruses in wild and domestic birds can help to recognize the sources of rotaviruses on industrial farms. Electron microscopy and PAGE could help to characterize virus morphology and dsRNA profile accurately.

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Conflicts of interest

The author declared no conflict of interest.

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ردیابی و تعیین ویژگی‌های مولکولی روتاویروس‌های پرندگان در مزارع گوشتی در گیلان

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چکیده

زمینه مطالعه: روتاویروس‌های پرندگان از عوامل مهم التهاب روده در پرندگان بوده و دارای گسترش جهانی می‌باشند. این ویروس‌ها نقش مهمی در سندروم کوتولگی و از رشد ماندگی در مرغ‌ها دارند.

هدف: هدف این مطالعه ردیابی و تعیین ویژگی‌های مولکولی روتاویروس‌های پرندگان در مزارع گوشتی استان گیلان می‌باشد. روش کار: در این مطالعه، ۱۰۰ نمونه از روده مرغان گوشتی سالم و بیمار در مزارع مختلف جمع‌آوری گردیدند و به وسیله روش RT-PCR بر روی ژن NSP۴ مورد آزمایش قرار گرفتند. برای تعیین ویژگی‌های ویروس‌های ردیابی شده، از توالی یابی ژن NSP۴ استفاده گردید.

نتایج: با استفاده از RT-PCR روتاویروس‌های پرندگان در ۴۶٪ نمونه‌ها ردیابی شدند. توالی یابی نسبی هفت ژن NSP۴ و آنالیز فیلوژنتیکی آن‌ها نشان داد که ویروس‌ها متعلق به گروه A روتاویروس‌های پرندگان بوده و درصد شباهت بین آن‌ها ۹۷/۵۲ تا ۱۰۰٪ است. شباهت توالی بین ویروس‌های این مطالعه و روتاویروس‌های مرغی قبلی ۳۱/۶۸ تا ۹۹/۵٪ بود. در مقایسه با روتاویروس بوقلمون (Ty-1)، و روتاویروس کبوتر، شباهت به ترتیب بین ۷۶/۹۸ تا ۷۸/۲۲٪ و ۷۴/۷۵ تا ۷۶/۹۸٪ نشان داده شد. تفاوت‌های مشخصی بین روتاویروس‌های این مطالعه و روتاویروس‌های انسان و خوک با تشابه ۳۱/۱۹ تا ۳۸/۸۶٪ برای ویروس‌های خوک و ۴۷/۷۷ تا ۴۸/۰۲٪ برای ویروس‌های انسان مشاهده شد.

نتیجه‌گیری نهایی: این مطالعه، برای اولین بار، ویژگی‌های مولکولی روتاویروس‌های پرندگان در ایران را تعیین نمود. نتایج این مطالعه نشان داد که گروه A روتاویروس‌های پرندگان یکی از پاتوژن‌های روده‌ای مهم در مرغ‌های گوشتی استان گیلان می‌باشند. مطالعه بیشتر بر روی ساختار کل ژنوم می‌تواند فرایند تکاملی روتاویروس‌های پرندگان در حال گردش در ایران را توضیح دهد.

واژه‌های کلیدی:

روتاویروس‌های پرندگان، مرغ گوشتی، ایران، ژن NSP۴، مطالعه فیلوژنتیک