



Molecular epidemiology of rotavirus A in Iranian broiler flocks

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ABSTRACT

Avian rotaviruses (RV) are still largely undefined despite being widespread in several avian species and significant economic the impact of rotavirus enteritis in poultry flocks. In this study, the presence of avian RV groups was investigated in 36 commercial poultry flocks with a history of enteric diseases in Iran. Intestinal contents of broiler chickens with diarrhea and stunting syndrome were analyzed by reverse transcription-polymerase chain reaction specific for RVs based on NSP4 gene. Through partial sequencing and BLAST analyses of 11 positive specimens, we identified avian-like RV group A (RVA) strain. There was high prevalence of group A rotaviruses (approximately 30%) in our samples. The phylogenetic analysis also revealed a close genetic relationship between the current isolates and other avian RVAs but located in the separate cluster. This study provides novel data on the prevalence of genetically different avian RVs in Iranian poultry flocks.

Key words: Broiler chicken, Iran, Phylogenetic analysis, Rotavirus A

Rotaviruses (RVs) have been recognized as a major cause of enteritis in a wide range of mammalian species including humans and avian species (McNulty and Reynolds 2008, Trojnar *et al.* 2009). Rotavirus, a member of the *Reoviridae* family, constitutes a triple-layered icosahedral protein capsid, and a genome made up of 11 segments of double-stranded RNA coding for six structural (VP1–4, VP6 and VP7) and 6 non-structural (NSP1–6) proteins (Buragohain *et al.* 2008). RVs are classified into 8 groups named A to H based on possession of different group antigens and terminal fingerprinting analysis of viral RNA and also VP6 nucleotide sequence identities (Ursu *et al.* 2011). NSP4 encoded by RNA segment 10 has multiple functions in rotavirus morphogenesis and pathogenesis. The NSP4 is an intracellular receptor on the rough endoplasmic reticulum during viral maturation. NSP4 has been shown to be as an enterotoxin, causing diarrhea in a mouse model system (Ito *et al.* 2001).

Rotavirus A (RVA) shows marked genetic diversity and

different genotypes of RVAs display peculiar distribution across various host species, indicating that host species barriers may exist. Despite host range restriction, spillover events of RVAs have been documented; striking examples include the transmissions of an avian RVA strain to cattle or the transmission of ruminant origin RVAs to ostrich (Ito *et al.* 2001). Birds are infected by group A avian rotaviruses (AvRV), which are found in both mammals and birds and by groups D, F and G that are found only in avian species (Bezerra *et al.* 2014). Traditionally, diagnosis of viral enteric infections in poultry has been made by electron microscopy (EM), immunofluorescent assays (IFA), and RNA-polyacrylamide gel electrophoresis (RNA-PAGE also called electrophoretotyping) to detect and identify the viruses and by ELISA to detect antibodies. Detection of rotavirus in intestinal contents or feces by reverse transcription PCR (RT-PCR) provides an alternate means of diagnosis and is specific and more sensitive than other tests (Bezerra *et al.* 2014). The current study aimed to detect AvRV in broiler farms and determine the genetic diversity among them based on sequencing and phylogenetic analysis of the genes encoding NSP4.

MATERIALS AND METHODS

Sampling: Intestinal contents (470) of 36 broiler flocks showing clinical signs of enteric disease were collected at the necropsy of culled or stunted chicks. Finally, 36 pooled samples were included in this study. Each pooled sample contained intestinal contents of 10–15 birds in each flock. All the 36 samples analyzed in this study were derived from

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poultry flocks that presented with one or more clinical signs and lesions associated with enteric diseases during 2014–2016 (two isolates in 2014, six isolates in 2015 and three isolates in 2016). Clinical manifestations mostly consisted of diarrhea, dehydration, anorexia, growth retardation and increased mortality. The age of birds was up to 3 weeks.

RNA extraction: Total RNA was extracted by using RNX plus™ kit (SinaClone, Iran) according to the manufacturer's instructions and stored in RNase-free water at -70°C .

RT-PCR for detection: For the reverse transcription (RT) reaction, 10 μl of template RNA and 1 μl of random hexamers (10 μM) were added and mixed. Then, the mixture was heated to 65°C for 5 min and quickly chilled on ice for 5 min. After brief centrifugation, 4 μl of RT buffer, 2 μl of dNTP mix (2.5 mM each), 1 μl of AMV reverse transcriptase (200 U/ μl), and 2 μl RNase-free water were added. The reaction mixture was incubated at 42°C for 60 min and then at 80°C for 5 min. The NSP4 gene was amplified (630 bp) using forward (NSP4-F30: 5'-GTG CGG AAA GAT GGA GAA C-3') and reverse (NSP4-R660: 5'-GTT GGG GTA CCA GGG A TTA A-3') primers (Roussan *et al.* 2012). The amplification was performed in a final volume of 20 μl containing 2 μl of distilled water, 13 μl of SinaClon PCR 2 \times master mix (SinaClon, Iran), 2 μl of primers (10 μM), and 3 μl of cDNA. The amplification was performed with a 35-cycle thermal profile (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).

Sequencing and bioinformatics analysis: An AccuPrep PCR purification kit (Bioneer Co., Korea) was used for the purification of the PCR products. Sequencing was performed with the NSP4-F30 and NSP4-R660 primers in both directions (Bioneer Co., Korea). Sequences were initially analyzed in Chromas PRO to confirm good quality read data. Pairwise sequence alignments were performed to determine nucleotide and amino acid sequence similarities. To determine the phylogenetic relationships, NSP4 gene sequence obtained in this study was compared with some sequences that are available in GenBank. Alignment and comparison of amino acid sequences were performed using Clustal W in MEGA 7.0 (Kumar *et al.* 2016). Distance-based neighbour-joining trees were constructed using the Tamura–Nei model (Tamura *et al.* 2011). The bootstrap values were determined from 1,000 replicates of the original data.

GenBank accession number of RVA sequence: The partial NSP4 gene sequences of Iranian RVAs were submitted to the GenBank data base under accession numbers MG922806 to MG922816.

RESULTS AND DISCUSSION

The economic significance of rotaviral enteritis to the poultry industry has not yet been defined (McNulty and Reynold 2008) but it is likely to be significant because they are suspected as the cause of several disorders in poultry such as enteritis, diarrhea, dehydration, weakness, anorexia, reduced feed conversion, weight gain rates, decreased

growth rate, runting and stunting syndrome (RSS) and lack of flock uniformity. Epidemiological information and genetic characterization of circulating RVs may be helpful in managing enteric disease outbreaks and the implementation of control measures in affected flocks. To the best of our knowledge, it is the first molecular detection and molecular characterization of avian rotaviruses circulating in poultry farms in Iran. We use NSP4 gene primers for detecting rotavirus groups in broiler flocks. All the samples in this survey were collected from sick chickens in the flocks with clinical signs of enteritis. We analyzed all RV isolates recovered during 2014–2016 based on nucleotide sequence diversity. Through partial sequencing and BLAST analyses, we identified avian-like rotavirus group A strain. Rotavirus A was detected in 30% (11/36 samples) of the samples by using RT-PCR and phylogenetic analysis. According to the results of 11 rotaviruses NSP4 gene sequencing, the identity range among avian RVs group A in Iranian broiler flocks was between 94.67 and 100%. Two isolates in 2014 were the same with 100% identity. The rate of homology among six isolates in 2015 was 95.38–99.83%. Three isolates in 2016 showed 96.77–99.83% identity to each other. Sequences of NSP4 obtained in this study shared the highest nucleotide identity to Nigeria and South Korea strains (85.53% and 84.92%, respectively) (Table 1).

The comparison of sequence analyses of NSP4s gene from chickens in this study and NSP4 gene from humans and pigs in Iran and other countries showed that avian and mammalian rotaviruses had separated at an early evolutionary stage and Iranian AvRVs are located in the separate cluster (Supplementary Figure 1). Group A rotaviruses have been isolated from mammals and birds, but so far group B, C, and E have been found only in mammals, and group D, F, and G have been detected only in birds (Bezerra *et al.* 2014). Interspecies transmission of rotavirus group A between birds and mammals and vice versa is probably rare. However, it had been reported earlier (McNulty and Reynold 2008).

Veen *et al.* (2016) detected two types of avian rotaviruses in Dutch broiler farms (group A: 96% and group D: 52%). In an investigation in Italy, Group Davian RV was detected in 107 of 117 samples tested (91.5%), whereas groups A, F, and G avian RVs were present in 70 (59%), 61 (52.1%) and 31 (26.5%) samples, respectively (Falcone *et al.* 2015). A molecular survey was performed to determine the presence of a broad range of enteric viruses including rotaviruses in intestinal samples derived from 34 commercial chicken flocks that experienced enteritis outbreaks between 2010 and 2012 in Korea. Diverse combinations of two or more enteric viruses were simultaneously identified in 51.7% of chicken farms positive for enteric viruses. The rate of positivity for RVs was 5.9% (Koo *et al.* 2013). Our research work was designed for detecting RVs in the flocks with clinical signs whereas further studies investigating co-circulation of different RV groups with specific primers in both symptomatic and asymptomatic chickens are needed

Table 1. Nucleotide acid sequence identity base NSP-4 gene between Iranian avian rotaviruses and some avian rotaviruses group A strains

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RVA_chicken-tc_JRL_Ch-1_1979(LC088136)																	
RVA_chicken_Japan_Ch-1_2000(AB065287)	100.00																
RVA_chicken_Germany_02V0002G3_2002(FJ169862)	97.47	97.47															
RVA_chicken_Nigeria_NIE11-075_2011(K932157)	94.25	94.25	95.70														
RVA_chicken_UnitedState_SEP-841_2005(EU400303)	93.70	93.70	93.50	90.82													
RVA_chicken_SouthKorea_AVRV-2_2011(UQ085408)	92.19	92.19	91.60	91.23	91.81												
RVA_Chicken-wt_BRA_AVE-73_2010(JX474761)	88.37	88.37	88.76	88.16	88.97	88.79											
RVA/chicken/Iran/H1063.5/2014	83.74	83.74	83.54	84.23	82.89	84.25	82.85										
RVA/chicken/Iran/H1063.7/2014	83.74	83.74	83.54	84.23	82.89	84.25	82.85	100.00									
RVA/chicken/Iran/H1204/2015	83.96	83.96	83.76	84.45	83.12	84.47	83.08	99.83	99.83								
RVA/chicken/Iran/H1485/2016	83.96	83.96	83.76	84.45	83.12	84.47	83.08	99.83	99.83	100.00							
RVA/chicken/Iran/H1546/2016	84.18	84.18	83.98	84.66	83.34	84.69	83.30	99.67	99.67	99.83	99.83						
RVA/chicken/Iran/H1429/2015	83.98	83.98	83.34	84.03	83.14	84.49	83.10	99.34	99.34	99.50	99.50	99.34					
RVA/chicken/Iran/H1205/2015	81.68	81.68	81.48	82.41	81.27	82.65	81.18	97.82	97.82	97.99	97.99	97.82	97.48				
RVA/chicken/Iran/H1428/2015	83.98	83.98	83.79	85.33	83.36	84.92	84.19	97.47	97.47	97.64	97.64	97.47	97.30	95.57			
RVA/chicken/Iran/H1425/2015	84.62	84.62	84.42	85.53	83.34	84.25	82.63	97.29	97.29	97.46	97.46	97.29	97.12	95.38	96.77		
RVA/chicken/Iran/H1426/2015	84.62	84.62	84.42	85.53	83.34	84.25	82.63	97.29	97.29	97.46	97.46	97.29	97.12	95.38	96.77	100.00	
RVA/chicken/Iran/H1468/2016	84.64	84.64	84.45	85.12	82.46	83.83	81.98	96.59	96.59	96.77	96.77	96.94	96.42	94.67	96.24	96.59	96.59

to assess the contribution of different factors related to bird susceptibility to RV infection. These findings will be useful to elucidate the epidemiology of avian RVs circulating in Iran and demonstrate RV potential involvement in enteric diseases of poultry.

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REFERENCES

- Bezerra D A M, da Silva R R, Kaiano J H L, de Souza Oliveira D, Gabbay Y B, Linhares A C and Mascarenhas J D. 2014. Detection, epidemiology and characterization of VP6 and VP7 genes of group D rotavirus in broiler chickens. *Avian Pathology* **43**(3): 238–43.
- Buragohain M, Cherian S S, Prabhakar G and Chitambar S D. 2008. VP6 capsid protein of chicken rotavirus strain CH2: sequence, phylogeny and *in silico* antigenic analyses. *Virus Research* **137**: 173–78.
- Falcone E, Busi C, Lavazza A, Monini M, Bertolotti M, Canelli E, Vignolo E, Ruggeri F M and Boniotti M B. 2015. Molecular characterization of avian rotaviruses circulating in Italian poultry flocks. *Avian Pathology* **44**(6): 509–15.
- Ito H, Sugiyama M, Masubuchi K, Mori Y and Minamoto N. 2001. Complete nucleotide sequence of a group A avian rotavirus genome and a comparison with its counterparts of mammalian rotaviruses. *Virus Research* **75**(2): 123–38.
- Koo B S, Lee H R, Jeon E O, Han M S, Min K C, Lee S B and Mo I P. 2013. Molecular survey of enteric viruses in commercial chicken farms in Korea with a history of enteritis. *Poultry Science* **92**(11): 2876–85.
- Kumar S, Stecher G and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**(7): 1870–74.
- McNulty M S and Reynolds D L. 2008. Rotavirus infections. *Diseases of Poultry*, pp 338–350. 12th edn. (Eds) Saif Y M, Fadly A M, Glisson J R, McDougald L R, Nolan L K and Swayne D E. Blackwell Publishing, Iowa.
- Roussan D A, Shaheen I A, Khawaldeh G Y, Totanji W S and Al-Rifai R H. 2012. Simultaneous detection of astrovirus, rotavirus, reovirus and adenovirus type I in broiler chicken flocks. *Polish Journal of Veterinary Sciences* **15**(2): 337–44.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S. 2011. MEGA5.1: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony method. *Molecular Biology Evolution* **28**: 2731–39.
- Ter Veen C, de Bruijn N D, Dijkman R and de Wit J J. 2017. Prevalence of histopathological intestinal lesions and enteric pathogens in Dutch commercial broilers with time. *Avian Pathology* **46**(1): 95–105.
- Trojnar E, Otto P and Johne R. 2009. The first complete genome sequence of a chicken group A rotavirus indicates independent evolution of mammalian and avian strains. *Virology* **386**(2): 325–33.
- Ursu K, Papp H, Kisfali P, Rigó D, Melegh B, Martella V and Bányai K. 2011. Monitoring of group A rotaviruses in wild-living birds in Hungary. *Avian Diseases* **55**(1): 123–27.