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Original Article

Characterization of reoviruses isolated from some broiler breeder flocks in Iran

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ABSTRACT

Avian reoviruses (ARVs) are considered as an important cause of several diseases in poultry, particularly arthritis and tenosynovitis. Tenosynovitis and arthritis, which are among the causes of chronic lameness in breeder flocks, can result in reduced egg production and culling of breeder hens. In this study, the molecular characteristics of ARVs in some broiler breeder flocks were investigated in Iran. After RNA extraction of the field samples, reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify two regions of ARVs for *S1* and *S4* genes. The positive samples were further analyzed by five restriction enzymes in restriction fragment length polymorphism (RFLP) for determining the strains. The phylogenetic analysis of *S1* and *S4* genes from the isolates indicated divergence into five and four major lineages, respectively. The sequence analysis of *S1* and *S4* genes of ARVs revealed that most of the positive samples were closely related to tenosynovitis-inducing ARVs (with less than 2% nucleotide divergence). Also, these samples were most homologous to S1133 strain, with 99.90% nucleotide and amino acid affinity.

Keywords: Molecular characteristics, Avian reoviruses, Tenosynovitis, Breeder flocks, Iran

Caractérisation des réovirus isolés à partir de poules pondeusesen Iran

Résumé: Les réovirus aviaires (ARVs) sont considérés comme une cause importante de maladies chez les volailles, particulièrement d'arthrite et de ténosynovite. Ces deux maladies sont à l'origine de boiteries chroniques chez les poules pondeuses,pouvant engendrer une réduction de la ponte et l'abattage des poules reproductrices. L'objectif de cette étude était la caractérisation moléculaire des ARVs affectant les poules pondeuses en Iran.Après l'extraction des ARN à partir des échantillons de terrain, une amplification en chaîne par polymérasetranscriptase inverse (RT-PCR) a été menée sur deux régions des ARVs, incluant les gènes S1 et S4. Les échantillons positifs ont été ensuite soumis à une analyse plus approfondie du polymorphisme de taille des fragments de restriction (PTFR) par 5 enzymes,afin de déterminer les souches impliquées. L'analyse phylogénétique des gènes S1 et S4 des isolats montre une divergence dans cinq et quatre grandes lignées, respectivement. L'analyse des séquences des gènes S1 et S4 a démontré que la majorité des échantillons positifs étaient étroitement liés aux ARVs induisant des ténosynovites (avec moins de 2% de divergence au niveau des nucléotides). De plus, ces échantillons étaient fortement homologues à la souche S1133, montrant une similitude nucléotidique et d'acides aminés de 99,90%.

Mots clés: Caractéristiques moléculaires, Réovirus aviaires, Ténosynovite, Poules pondeuse, Iran

INTRODUCTION

Avian reoviruses (ARVs) constitute an important cause of poultry diseases, such as arthritis, tenosynovitis, chronic respiratory disease, and malabsorption syndrome (Dale et al., 1983). These viruses include 10 segments of double-stranded RNA (Benavente and Martinez-Costas, 2007), as well as eight structural and four non-structural proteins encoded by ARV genome (Shapouri et al., 1995). The sigma C protein is encoded by *S1* gene (Shapouri et al., 1995). Sigma NS is another ARV protein, which is encoded by *S4* gene (Chiu and Lee, 1997). Different methods have been used for the identification of ARVs, such as immunodiffusion, virus neutralization, enzyme-linked immunosorbent assay (ELISA), *in situ* hybridization, and immunoblot assays (Kant et al., 2003). ARV isolates can rapidly evolve, resulting in a higher heterogeneity and pathogenicity in neutralizing antigens, as well as considerable cross-reactions among the heterologous types (Clark et al., 1990). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and phylogenetic analysis have been established to characterize the new-field and standard isolates of ARV (Liu et al., 2004). It has been shown that the S1 and S4 segments of ARV genome exhibit higher variability than other segments of the ARV genome (Li et al., 2003; Liu et al., 2004). The genetic divergence enables researchers to rapidly differentiate ARV isolates, based on the restriction profiles of S1 and S4 genomic segments. In this regard, in a previous study in Iran, reverse transcription (RT)-PCR and nested PCR of *S1* gene were performed for the first time to confirm the presence of ARVs in tissue samples, obtained from suspicious flocks in some provinces of Iran; the findings showed only one positive sample out of 28 studied samples (Harzandi et al., 2006). For years, many Iranian breeder flocks have been vaccinated with reovirus vaccines; S1133 is also the most commonly used vaccine strain. In this study, we aimed to detect ARV directly from fecal swab samples, collected from broiler breeder flocks which had not been vaccinated with reovirus vaccines. By performing RT-PCR and analyzing the phylogenetic relationships, we characterized the molecular relationship of ARVs with other reoviruses which have been already characterized in other parts of the world.

MATERIALS AND METHODS

Viruses. Based on the RT-PCR results, 120 pooled fecal swab samples were randomly collected from some broiler breeder flocks, which had not been vaccinated and had no clinical signs of arthritis or tenosynovitis. In our previous study on these breeder flocks in central and northern provinces of Iran, five and six positive samples for S1 and S4 segments were detected, respectively (Hedayati et al., 2013). The RT-PCR amplified products of both *S1* and *S4* genes were cleaved by five different restriction enzymes (RE) (i.e., BcnI, HaeIII, TaqI, DdeI, and HincII) to produce identical patterns among the isolates. All RE profiles of the isolates were compatible with S1133 and 750505 strains for both segments (Hedayati et al., 2013). Two ARV field isolates positive for *S1* (S1AR03IR and S1AR04IR) and two ARV field isolates positive for *S4* (S4AR01IR and S4AR02IR) genes were included in this study. The samples were prepared as described in the literature (Zhang et al., 2006). Briefly, the fecal swab samples were separately placed in a sterile tube, containing phosphate-buffered saline (PBS), and were transferred to a laboratory under proper conditions. In the laboratory, every six fecal swab samples were pooled and centrifuged at 3000x g for 5 min. Then, each supernatant was harvested and filtered through a sterile 0.45 µm membrane filter (Orange, USA). The filtered supernatants were collected in a 1.5 ml sterile RNase- and DNase-free microtube and stored at -20˚C until further use as a substrate in the RNA extraction protocol.

RNA extraction and RT-PCR. A commercial kit (High Pure Viral RNA Kit, Roche, Switzerland) was used for the extraction of viral RNA from the fecal samples. For cDNA synthesis, a commercial kit (two-step RT-PCR kit, RTPL12, Vivantis, Malaysia) was used to prepare cDNA from the extracted RNA

(Razmyar and Peighambari, 2008). The full-length cDNA of each ARV isolate was amplified by appropriate primer pairs, which were selected based on the cDNA sequences of genomic S1 and S4 segments of ARV S1133 (Wickramasinghe et al., 1993; Shapouri et al., 1995). To amplify the *S1* gene (1023 bp), two primer pairs (S1A and S1H) were used. In addition, two primer pairs (S4-p4 and S4-p5) were used to amplify the *S4* gene (437 bp) of the standard and new-field strains (Liu et al., 2004; Bruhn et al., 2005). Amplification was carried out in a thermocycler (Mini Bio-Rad Mastercycler, USA). Amplification was detected by gel electrophoresis (Apelex, France) in 1.2% Agarose gel in tris-acetate-EDTA (TAE) buffer. The PCR amplification and gel electrophoresis of the PCR products were carried out as described in the literature (Hedayati et al., 2013). The primers and other materials used in the PCR reaction were provided by CinnaGen Inc., Iran.

Sequencing and phylogenetic analysis. The PCR products from S1 (1023 bp) and S4 (437 bp) segments were purified, using the Roche purification kit. The products were submitted to Eurofins MWG Operon Company (Germany) for automated sequencing in both directions, using PCR primers as the sequencing primers. The nucleotide and predicted amino acid sequences were aligned with the Clustal alignment algorithm. The phylogenetic analysis based on the nucleotide and amino acid sequences was conducted, using the distance method, unweighted pair group method with arithmetic mean (UPGMA), and calculation of bootstrap values for 1000 replicates, using MEGA software version 4.0 (Tamura et al., 2007).

RESULTS

The results of the sequence analysis of S1AR03IR and S1AR04IR isolates were compared with the published sequences of 750505, 176, and 1733 ARV strains at nucleotide and amino acid levels. The two Iranian isolates, i.e., S1AR03IR and S1AR04IR, were 100% identical to S1AR03IR and S1AR04IR isolates in terms of nucleotide and amino acid positions, whereas they differed from other strains in several positions (Tables 1 & 2). The nucleotide and amino acid sequences for S4AR01IR and S4AR02IR isolates were also 100% similar, while they differed from the published sequences of 601s1 and 176 ARV strains in some positions (Tables 3 & 4). The phylogenetic analysis of the selected ARV strains in the phylogenetic tree showed five clusters in the *S1* gene. The isolated strains in this study (S1AR03IR and S1AR04IR) were classified in the same cluster as common ARV strains such as S1133, 750505, 2408, 139, and 176 strains, while 916 and 918 strains, S1 segment of turkey-origin reoviruses, and ARV Chinese strain (GX 2012) were in different clusters (Figure 1). The phylogenetic analysis of the *S4* gene of the Iranian ARV isolates (S4AR01IR and S4AR02IR) indicated four major lineages, which were in the same cluster as S1133, 176, 750505, 1733, and 2408 strains. Other strains including 1017, 918, 138, and R2 (Taiwanese ARV strains), as well as the Chinese duck reovirus (DRV-GZ) strain, were from different lineages (Figure 2). The sequence alignment and phylogenetic analysis indicated that S1AR03IR, S1AR04IR, S4AR01IR, and S4AR02IR isolates exhibited the highest homology and were most closely related to the ARV reference strain, S1133. The sequence analysis of the amplified segments of *S1* and *S4* genes revealed that the strains S1AR03IR, S1AR04IR, S4AR01IR, and S4AR02IR were closely homologous to most ARV strains (less than 2% nucleotide divergence).

DISCUSSION

In this study, the results of the sequence analysis of the isolates, S1AR03IR and S1AR04IR, showed 99.26%, 99.26%, 98.08%, and 99.89% nucleotide homology to the published sequences of Fahey vaccine strain, S1133WT vaccine strain, vaccinal VA, and P100 vaccine strain, respectively. In a previous study, the sequencing of ARV isolates from Chinese poultry flocks revealed a high homology between Hb06 and ARV reference strain, S1133, with 98.97% nucleotide

identity (Pu et al., 2008). In this study, the strains S1AR03IR, S1AR04IR, S4AR01IR, and S4AR02IR revealed high homology to S1133, with 99.90% nucleotide identity, while the homology of S1AR03IR and S1AR04IR strains to Hb06 was 99.26%. Also, the sequence analysis of Hb06 indicated 97.24% homology to Fahey vaccine strain and 98.16% homology to P100 vaccine strain. In addition, the S1AR03IR and S1AR04IR strains showed 99.26% and 99.89% homology to Fahey vaccine and P100 vaccine strains, respectively. In a previous study performed in China, the avian, duck, and goose reovirus RNAs were detected in the cell culture supernatant and the clinical samples through RT-PCR amplification of a 598 bp product from the σA(S2)-encoding gene. The nucleotide and amino acid sequence identities in the amplified σA-encoding gene among duck and chicken species were 74.2–78.4% and 86.9–92.0%, respectively (Zhang et al., 2006). The S1AR03IR and S1AR04IR strains showed 99.26% homology to the American ARV176 strain, and S4AR01IR and S4AR02IR strains exhibited 99.41% homology. The homology of DRV strains to S1AR03IR and S1AR04IR was 99.79%, while the homology to S4AR01IR and S4AR02IR was 81.53%. In a previous study, the primers used from highly conserved regions of *S2* and *S4* genes confirmed four ARV vaccine strains (1133, 1733, 2408, and Olson WVU2937 strains), two ATCC strains (VR826 and VR856), as well as several ARV field isolates obtained from domestic, wild, and pet birds (Bruhn et al., 2005). The results indicated that 55% and 80% of the 64 ARV field isolates could be detected, using ARV S2 RT-PCR and ARV S4 RT-PCR, respectively (Bruhn et al., 2005). In addition, 11% of the field isolates were not detectable by ARV S2 and S4 RT-PCR (Bruhn et al., 2005). In the present study, the identity of the amplified products and their characterization were further confirmed by four restriction enzymes, i.e., DdeI, RsaI, PvuI, and HincII. In a previous study, the nucleotide divergence in the S-class genome segment of ARV strains was less than 10% (Li et al., 2003). The phylogenetic analysis of σC-encoding gene showed six clusters, while the other S-class genes diverged into two to four distinct lineages (Pu et al., 2008). According to the literature, the phylogenetic analysis of the nucleotide sequences of σC- and σNS-encoding genes was indicative of six distinct lineages in the σC tree and five distinct lineages in the σNS tree (Banyai et al., 2011). However, in this study, the phylogenetic analysis of *S1* and *S4* genes of the Iranian ARV isolates indicated five and four major lineages, respectively (Banyai et al., 2011). In a study in Taiwan, the full-length of σC-encoding and σNS-encoding genes of ARV was amplified through RT-PCR, resulting in 1022 and 1152 bp fragments, respectively (Liu et al., 2004); based on the findings, vaccine strains and several field isolates could be detected. The amplified products were then digested with five different restriction enzymes, i.e., BcnI, HaeIII, TaqI, DdeI, and HincII, respectively. The restriction fragment profiles demonstrated heterogeneity between the vaccine and Taiwanese isolates (Liu et al., 2004). The ARV field isolates also showed different RE digestion patterns, which classified them into four distinct groups by patterns observed on the amplified products of σC-encoding gene. Interestingly, the phylogenetic tree based on the nucleotide sequences of σC-encoding gene revealed that Taiwanese ARV isolates could be classified into four distinct groups. Therefore, genotyping was consistent with typing, based on the RFLP of the σC-encoding gene (Liu et al., 2004). In our earlier study (Hedayati et al., 2013), by using the same digestion enzyme (Li et al., 2003), we were able to differentiate the ARV isolates. More recently, it has been suggested that the S-class genome segments show higher rates of synonymous substitutions than those of non-synonymous substitutions, with the exception of the σC-encoding gene of ARV in which the rates of non-synonymous substitutions are higher than those of synonymous substitutions (Hsu et al., 2005). In a previous study, sequencing based on the σC gene in Tunisian ARV isolates was closely related to the

Table 1. Nucleotide differences at certain positions in the S1 sequence of the selected avian reovirus (ARV) strains compared with S1AR03IR and S1AR04IR[a].

Strain/ isolate	Nucleotide position												
	116	214	215	319	340	356	405	407	498	550	624	736	912
S1AR03IR	A	T	A	G	T	G	A	A	G	T	G	C	T
S1AR04IR	.	.	.	.	.	.	.	.	.	.	.	.	.
176	.	.	C	C	C	A	G	C	.	.	.	.	.
1733	.	C	.	.	.	.	.	.	C	.	.	.	.
750505	T	.	.	.	.	.	.	.	.	C	C	T	C

[a]Dots indicate sequences identical to those of the S1AR03IR strain.

Table 2. Amino acid differences at certain positions in the S1 sequence of the selected avian reovirus (ARV) strains compared with S1AR03IR and S1AR04IR[a]

Strain/ isolate	Amino acid							
	2	17	49	84	91	113	161	193
S1AR03IR	N	N	N	R	I	I	S	R
S1AR04IR	.	.	.	.	.	.	.	.
176	T	.	.	T	T	V	.	.
1733	.	.	I	.	.	.	.	.
750505	.	I	.	.	.	.	P	P

[a]Dots indicate sequences identical to those of the S1AR03IR strain.

Table 3. Nucleotide differences at certain positions in the S4 sequence of the selected avian reovirus (ARV) strains compared with S4AR01IR and S4AR02IR

Strain/ isolate	Nucleotide position				
	45	212	262	273	311
S4AR01IR	C	A	C	C	G
S4AR02IR	.	.	.	.	.
176	.	.	T	T	A
601s1	G	G	C	C	.

[a]Dots indicate sequences identical to those of the S4AR01IR strain.

Table 4. Amino acid differences at certain positions in the S4 sequence of the selected avian reovirus (ARV) strains compared with S4AR01IR and S4AR02IR[a]

Strain/ isolate	Amino acid	
	71	72
S4AR01IR	R	D
S4AR02IR	.	.
176	.	.
601s1	H	N

[a]Dots indicate sequences identical to those of the S4AR01IR strain.

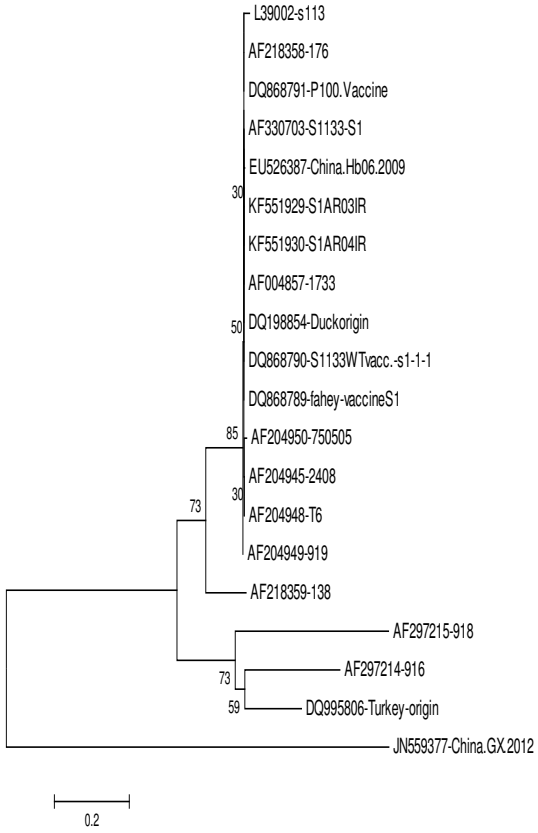

Figure 1. The phylogenetic tree of the selected avian reovirus (ARV) strains based on the nucleotide sequence of a segment of *S1* gene. The branched distances correspond to a sequence divergence (numbers below the branches indicate the bootstrap value from 1000 bootstrap replicates).

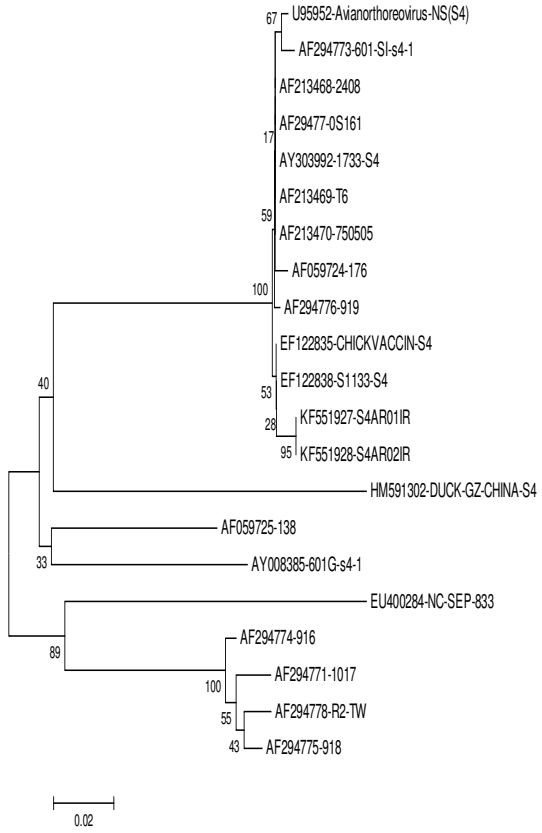

Figure 2. The phylogenetic tree of the selected avian reovirus (ARV) strains based on the nucleotide sequence of a segment of *S4* gene. The branched distances correspond to a sequence divergence (The numbers below the branches indicate bootstrap value from 1000 replicates).

American strain, S1133. Analysis of the whole σB-encoding gene was performed to allow for the rapid detection of ARV infections with different Tunisian isolates. The phylogenic comparison of genotype clustering based on the nucleotide sequences of σC gene from Tunisian isolates showed that they were closely related to each other and could be classified in cluster I. Therefore, a close relationship with S1133 was detected, similar to most ARV strains. In addition, in the mentioned study, alignment of the σC-encoded gene nucleotides of all the identified strains was observed. Also, for the σB gene sequences, the phylogenic tree showed that the Tunisian strains were relatively close to each other and could be classified in cluster I (Kort et al., 2013). In the present study, the homology of S1AR03IR, S1AR04IR, S4AR01IR, and S4AR02IR to the strain S1133 was the highest (with 99.90% nucleotide and amino acid identity), and the strains were grouped in the same cluster (cluster I). In this study, the constructed phylogenetic trees showed that the σC- and σNS-encoding genes evolved into five and four lineages, respectively; however, this finding was inconsistent with previous studies (Yin and Lee, 1998; Kant et al., 2003; Liu et al., 2004; Banyai et al., 2011; Kort et al., 2013). It was concluded that the classification of ARV could be implemented, using the σC gene, which is the most variable identified gene. In the meantime, the sequence analysis of *S1* and *S4* genes of ARVs revealed that the positive samples were closely related to most tenosynovitis-inducing ARV strains (with less than 2% nucleotide divergence). Also, they showed the highest homology to the strain S1133 (99.90% nucleotide and amino acid identity). In the current study, the phylogenetic tree showed five and four clusters for the *S1* and *S4* genes of ARVs, respectively. Although the results of the present study are related to three provinces, which contain the highest number of breeder flocks in Iran, the same results could be expected from other parts of the country. Nevertheless, similar studies in other provinces are recommended.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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