Detection of avian reoviruses causing tenosynovitis in breeder flocks in Iran by reverse transcription-polymerase chain reaction (RT-PCR) and restriction enzyme fragment length polymorphism (RFLP)

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Abstract:

BACKGROUND: Avian reoviruses (ARVs) are members of the Orthoreovirus genus; one of the 12 genera of the Reoviridae family. The ARVs are the cause of some important diseases in poultry such as reovirus-induced arthritis, tenosynovitis, chronic respiratory disease, and mal-absorption syndrome. **OBJECTIVES:** In this study, the presence of ARVs in the Iranian breeder flocks was investigated through reverse transcription-polymerase chain reaction (RT-PCR) and restriction enzyme fragment length polymorphism (RFLP). **METHODS:** A total of 800 fecal swab samples were initially collected from breeder flocks (older than 45 weeks of age). They were then sent to the laboratory in containers with PBS, and after that they were pooled and finally to 120 samples were obtained. The total RNA extracted from the pooled fecal samples were used to amplify the selected parts of the S1 (1023 bp) and S4 (437 bp) genes from the ARV field isolates using RT-PCR. The positive RT-PCR amplified products were further analyzed by RFLP using five restriction enzymes. **RESULTS:** Based on the findings, 5 samples were positive with the S1 primer and 6 samples were with the S4 one. The patterns observed after the digestion of PCR products revealed that the isolates of this study were identical to both the S1133 vaccine and standard strains. **CONCLUSIONS:** The findings suggested that the RT-PCR/RFLP analysis might be considered as a simple and rapid approach for the differentiation of ARV isolates. This study was the first molecular detection of the ARVs presence in the Iranian breeder flocks using the RT-PCR amplification of the S1 and S4 genes and RFLP analysis.

Introduction

Avian reoviruses (ARVs) are the cause of some important disorders in poultry. In particular, reovirus-induced arthritis, chronic respiratory disease, and malabsorption syndrome may be associated with considerable economic losses (Hieronymus et al., 1982). The genome of ARVs is composed of 10 segments of double-stranded RNA (Benavente et al., 2007). The ARVs genome encodes 12 proteins including eight structural and four non-structural proteins (Varela et al., 1994). Among the ARV proteins, the σC protein, encoded by the ARV S1 gene (Varela et al., 1994; Shapouri et al., 1995), has 326 amino acids in length and possesses both specific and broadly-specific epitopes (Wickramasinghe et al., 1993; Shapouri et al., 1996). Another protein of ARV, σNS, encoded by the S4 gene (Chiu et al., 1997), has been reported for its single-stranded RNA binding activity (Yin et al., 1998; Benavente et al., 2007).

Although the pathological effects of ARVs in poultry have been extensively studied, relatively little is known about the variations and evolution of the ARV genes.

Various methods used to identify ARV isolates and detect antibodies against the ARV include immunodiffusion, virus neutralization, enzyme linked immunosorbent assay (ELISA), in situ hybridization, and immunoblot assays (Lee et al., 1994; Liu et al., 1999, 2002, 2003; Kant et al., 2003). Due to the influence of various factors such as standardization of the procedure and the method of antiserum production on serological analyses of viruses, more tests with monoclonal antibodies raised against different serotypes are needed to confirm the presence of truly distinct serotypes. Furthermore, ARV isolates can rapidly evolve, resulting in a wider heterogeneity in pathogenicity and their neutralizing antigens, as well as considerable cross-reaction among the heterologous types (Rosenberger et al., 1989; Clark et al., 1990). In order to find a more practical and accurate method of identifying ARV isolates, PCR-RFLP and phylogenetic analysis were conducted to characterize the new field isolates of ARV (Liu et al., 2003, 2004). In recent years, molecular-based methods were effective in the characterization of viruses (Lin et al., 1991; Liu et al., 1999). The RT-PCR is able to detect as little as 1 pg of RNA and the detection of such a small amount of RNA may enable the viral RNA to be amplified directly from clinical and environmental samples (Xie et al., 1997). It has been shown that the segments S1 and S4 of the ARV genome express a higher variability compared to the other segments of the ARV genome (Liu et al., 2003, 2004). Genetic divergence enabled researchers to rapidly differentiate ARV isolates based on the restriction profiles of the S1 and S4 genomic segments. In this investigation, we attempted to detect avian reoviruses directly from the fecal swab samples collected from the breeder flocks of Iran through the RT-PCR and differentiate the ARV field isolates based on the restriction enzyme fragment length polymorphism (RFLP) patterns.

Materials and Methods

Sampling: A total of 800 fecal swab samples were collected from the breeder flocks in three provinces (i.e. Mazandaran, Gilan, Ghazvin) of Iran. The size of the flocks varied from 20000 to 40000 hens. Every fecal swab sample was placed in a sterile tube containing Phosphate Buffer Saline (PBS) and then transferred to our laboratory in cold conditions. In the laboratory, every 6-7 fecal swab samples were pooled and centrifuged for 5 min at 3000 x g. Then, each supernatant was harvested and filtered through a sterile membrane filter (Orange®, Belgium) with a 0.45 µm pore size. Each of them was collected in a 1.5 mL sterile RNase and DNase free microtube and stored at -20°C until future use (Zhang et al., 2006).

RNA extraction: A commercial kit (High Pure Viral RNA Kit®, Roche, Germany) was used to extract the total RNA from the fecal samples prepared as described before. Briefly, 200 µL of each prepared sample was added to a 1.5 mL sterile RNase and DNase free microtube which contained 400 µL of working solution (polyA plus binding buffer) and then processed as recommended by the manufacturer. The extracted viral RNA was stored at -70°C until further use.

Reverse transcription-polymerase chain reaction (RT-PCR): To make cDNA, a commercial cDNA synthesis kit (2-steps RT-PCR kit, RTPL12®, vivantis, Malaysia) was used. The positive control was the live vaccine S1133 strain. The procedure recommended by the manufacturer was utilized with some modifications. Briefly, 8 µL RNA extracts, 1 µL Random Hexamer primer, and 1 µL dNTPs were added to a 0.2 mL microcentrifuge tube, boiled for 4 min, and then cooled on ice for 2 min. Two µL 10x RT buffer plus 1 µL M-MULV RT enzyme (200 u/µL), and 7 µL Nuclease-free water were added to the previous mixture, then it was incubated for 10 min at 25°C, one hour at 42°C, 5 min at 85°C, and finally cooled on ice, and stored at -20°C.

To amplify the full-length cDNA of the ARV isolates, appropriate pairs of the primers were chosen based on the cDNA sequences of the genomic S1 and S4 segments of ARV S1133 (Shapouri et al., 1995; Chiu et al., 1997). For the S1 gene, two pairs of primer S1A (5`-CTTGTCTTATAGTTCATTGGG- 3`, identical to nucleotides 601 to 621) and S1H (5`-TCCCAGTACGGCGCCACACC-3`, complementary to nucleotides 1622 to1603) were used to amplify the S1 gene (1023 bp) of live S1133

vaccine strain and the field isolates used in this study (Liu et al., 2003). The amplification was carried out in a 50-µL reaction volume containing 5 µL 10x PCR buffer, 1 µL 10 mM dNTPs, 1.25 µL of each primer (10 pmol/µL), 0.25 µL Taq DNA polymerase (5U/µL), 1.5 µL 50 mM MgCl2, 33.75 µL of dH2O, and 6 µL cDNA dilution. The thermocycler (Mini BIO RAD® Mastercycler) used for the amplification was programmed as follows: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 60°C for 45 sec, 72°C for 65 sec, and a final extension at 72°C for 10 min. In all of the PCR reaction sets, negative controls (dH2O instead of cDNA) were included.

For the S4 gene, two pairs of primer S4-p4 (5`-GTGCGTGTTGGAGTTC3`) and S4-p5 (5`-ACAAAGCCAGCCAT(G/A)AT-3`) were used to amplify the S4 gene (437 bp) of the live S1133 vaccine strain and the field isolates used in this study (Liu et al., 1999, 2004; Bruhn et al., 2005). The amplification was carried out in a 25-µL reaction volume containing 2.5 µL 10x PCR buffer, 0.5 µL 10 mM dNTPs, 1 µL of each primer (10 pmol/µL), 0.2 µL Taq DNA polymerase (5U/µL), 0.75 µL 50 mM MgCl2, 15.05 µL of dH_2O, and 4 µL cDNA dilution. The thermocycler was programmed as follows: 94°C for 4 min followed by 35 cycles of 94°C for 30 sec, 54°C for 45 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. In all the PCR reaction sets, negative controls (dH2O instead of cDNA) were included.

The amplification products for both S1 and S4 genes were detected by gel electrophoresis (Apelex, France) in 1.2% agarose gel in the TAE buffer. The gels were run for 55 min at 80 V, stained with DNA SAFE STAIN® or CYBER SAFE® (1 µL/30 mL agarose gel), exposed to ultraviolet light, and photographed at the end (Visi-Doc-It system, UVP, UK). A commercial 100-bp DNA ladder (Vivantis, Malaysia) was used as the molecular-weight marker in each gel running. The primers and other materials used in the PCR reaction and the gel electrophoresis were provided by Cinnagen Co. (Iran).

Restriction enzyme fragment length polymorphism (RFLP): The amplification products were digested with five different restriction enzymes (RE) including BcnI, HaeIII, TaqI (Fermentas Life Science, Germany), DdeI (Roche, Germany), and HincII (Vivantis, Malaysia) according to the manufacturers' instructions. Briefly, 8 µL PCR product, 2 µL RE buffer, 1 µL RE, and 14 µL dH2O were mixed, incubated at 37°C (at 65°C for TaqI) for 2.5 h, and run in 1.5% agarose gel at 7 v/Cm for 60 min. The DNA fragments on agarose gels were stained with CYBER SAFE® (Cinnagen), visualized by ultraviolet illumination, and photographed at the end. All reactions were undertaken in duplicates (Shapouri et al., 1995; Chiu et al., 1997).

Result

RT-PCR: Among the 120 pooled fecal samples, 5 samples were positive for the S1 segment (Figure 1) and 6 samples were positive for the S4 segment (Figure 2).

Restriction enzyme fragment length polymorphism (RFLP): The digestion of the PCR products by the five restriction enzymes resulted in different patterns which were compared with the restriction sites and patterns of the S1133 vaccine strain and other known strains (originated from tenosynovitis) whose sequence data are available in the GenBank data (Table 1).

The RT-PCR amplified products of the S1 gene were cleaved by TaqI into two fragments of 430 and 880 bp (Figure 3.a, lanes 1, 2, and 3), by DdeI into one fragment of 480 bp (Figure 3.c, lanes 1, 2, and 3), and by HincII into two fragments of 320 and 620 bp (Figure 3.d, lanes 1, 2, and 3). BcnI (Figure 3.b, lanes 1, 2, and 3), and HaeIII (Figure 3.e, lanes 1, 2, and 3) did not cut the S1 gene amplified products. All five positive samples for the S1 gene demonstrated identical patterns and were compatible with the S1133 vaccine strain and S1133 standard and 750505 strains.

The RT-PCR amplified products of the S4 gene were cleaved by TaqI, DdeI, and HaeIII into one fragment of 350 bp (Figure 3.a, lanes 4, 5, and 6), 300 bp (Figure 3.c, lanes 4, 5, and 6), and 120 bp (Figure 3.e, lanes 4, 5, and 6), respectively. BcnI (Figure 3.b, lanes 4, 5, and 6) and HincII (Figure 3.d, lanes 4, 5, and 6) did not cut the S4 gene amplified products. All six positive samples for the S4 gene demonstrated identical patterns and were compatible with the S1133 vaccine strain and S1133 standard and 750505 strains.

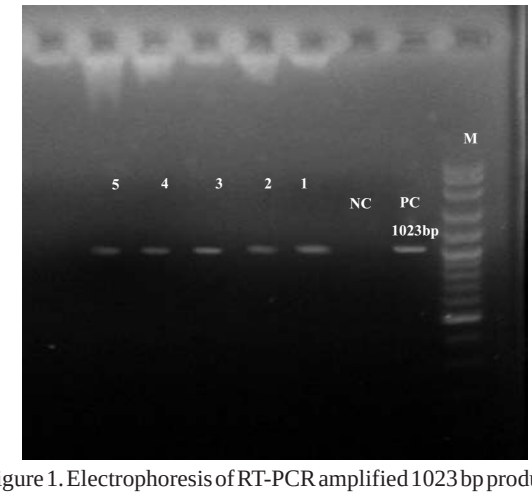

Figure 1. Electrophoresis of RT-PCR amplified 1023 bp products of the S1 gene on 1.2% agarose gel and stained with safe stain (cyber green®). Amplified 1023 bp bands of field isolates are shown in lanes 1 to 5. Lanes M, PC, and NC indicate 100 bp ladder, positive control (S1133 vaccine strain), and negative control (dH2O instead of cDNA), respectively.

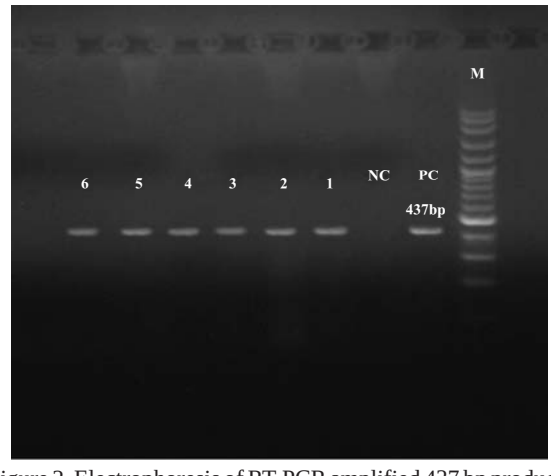

Figure 2. Electrophoresis of RT-PCR amplified 437 bp products of the S4 gene on 1.2% agarose gel and stained with cyber green®. Amplified 437 bp bands of field isolates are shown in lanes 1 to 6. Lanes M, PC, and NC indicate 100 bp ladder, positive control (S1133 vaccine strain), and negative control (dH2O instead of cDNA), respectively.

Discussion

Avian reoviruses (ARVs) have been implicated in causing many disease syndromes, especially arthritis/tenosynovitis syndromes which are not distinguishable from other poultry diseases by clinical examination. In such circumstances laboratory diagnosis of the disease is therefore required. Due to problems associated to serological procedures in analyzing the ARVs, molecular analysis methods have been developed for the identification and characterization of the ARV isolates. The PCR-RFLP and phylogenetic analysis have been conducted for the differentiation of the standard and new field isolates of the ARVs (Lin et al., 1991; Liu et al., 1999). The segments S1 and S4 of the ARV genome have been found to be suitable for differentiating the ARV isolates and studing genomic variations due to the presence of high variability in these regions (Liu et al., 2003, 2004). The digestion of the RT-PCR products of the amplified S1, S3, and S4 gens by restriction enzymes (RE) have shown variations among the isolates (Lee et al., 1998; Liu et al., 2004).

There are many recent reports regarding the avian reoviruses infections in poultry flocks around the world. A sero-prevalence study on the Nigerian poultry flocks revealed that the prevalence of anti-reovirus antibody was 41% (Owoade et al., 2006). In China, a sero-epidemiologic study of ARV infections from egglaying chicken flocks showed that 92% (542/587) of the average positivity (Pu et al., 2008). The same authors isolated an ARV from the flocks with suspicious ARV infections and confirmed the ARV by PCR. The sequence of the ARV isolates revealed high homology with the vaccine strain S1133, with a 98.97% nucleotide identity (Pu et al., 2008). In USA, the presence of the ARV field isolates among the US poultry flocks was confirmed by the nested-PCR that amplified the S1 gene segment. The further sequence analysis of the isolates revealed that the US isolates were closely related, but different from the Australian isolates (Liu et al., 1997). In another US study among chicken and turkey flocks, 4 samples from chickens and 8 samples from turkeys were positive in the RT-PCR of the S4 segment gene (Pantin-Jackwood et al., 2008). In China, Zhang et al., (2006) detected the avian, duck, and goose reovirus RNA RT-PCR amplification of the σA (S2) encoding gene. The nucleotide and amino acid sequence identities in the amplified σA-encoding gene were 74.2-78.4% and 86.9-92.0%, respectively, between duck/goose and chicken species (Zhang et al., 2006). In another study, Liu et al., (1999) used a nested RT-PCR with subsequent restriction endonuclease analysis for the identification of the sigma C-encoded gene (S1) ARVs. PCR products derived from the S1 gene of all tested ARVs resulted in a specific DNA band of 1023 bp, indicating that there were no

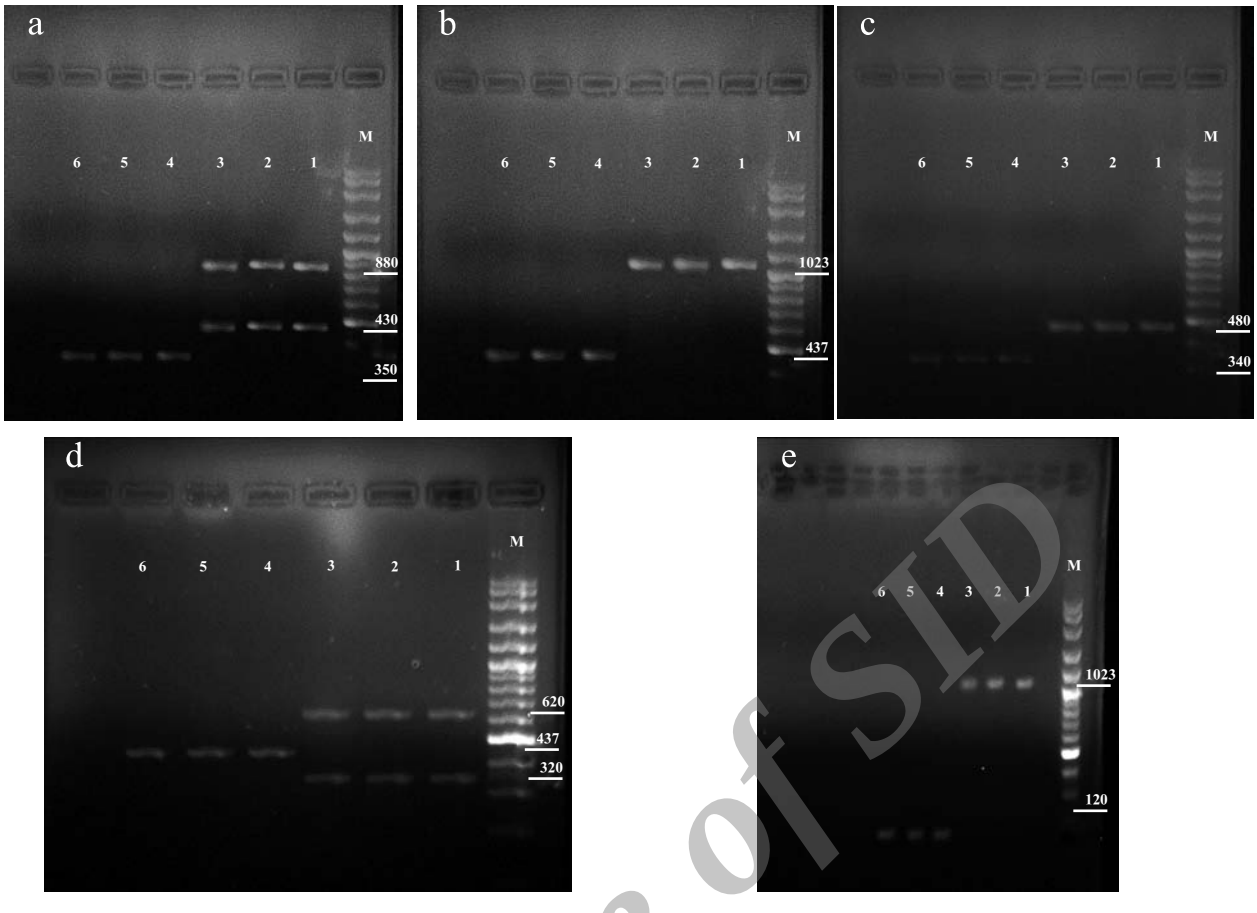

Figure 3. Electrophoresis of the PCR products from the S1 gene (1023 bp) and S4 gene (437bp) of ARV field isolates digested with five restriction enzymes: TaqI (a), BcnI (b), DdeI (c), HincII (d), and HaeIII (e). Lane M illustrates the 100 bp ladder. Lanes 1, 2, and 3 indicate the 1023 bp PCR product from the S1 gene of S1133 (standard strain) and two field ARV isolates, respectively. Lane 4, 5, and 6 indicate the 437 bp PCR product from the S4 gene of S1133 (standard strain) and two field ARV isolates, respectively.

apparent insertions or deletions in this region. Our findings with RT-PCR of the S1 gene segment were comparable with those presented by Liu et al., (1999). The PCR amplified cDNA fragment (1023 bp) cleaved with two generated 565 and 458 bp PstI fragments (Liu et al., 1999). Bruhn et al., (2005) used primers from highly conserved regions of the S2 and S4 genes and confirmed four ARV vaccine strains (i.e. 1133, 1733, 2408 and Olson WVU2937) and two ATCC strains (i.e. VR826 and VR856), as well as several ARV field isolates were obtained from domestic, wild, and pet birds. They reported that 55% and 80% of the 64 ARV field isolates were detected with the ARV S2 RT-PCR and ARV S4 RT-PCR, respectively. However, 11% of the field isolates were not detected by the ARV S2 and S4 RT-PCR. The identity of the amplified products was further confirmed by restriction enzymes (i.e. DdeI, RsaI, PvuI and HincII) (Bruhn et al., 2005). In a Taiwanese study (Liu et al., 2004), the full-length σC-encoding and σNS-encoding genes of avian reovirus (ARV) were amplified using the RT-PCR that resulted in the fragments of 1022 and 1152 base pairs, respectively. In that study vaccine strains and several field isolates were also detected. The amplified product was then digested with five different restriction enzymes (i.e. BcnI, HaeIII, TaqI, DdeI, and HincII). The restriction fragment profiles demonstrated heterogeneity between the vaccine and Taiwanese isolates. The ARV field isolates also showed different RE digestion patterns so that they could be classified into four distinct groups based on the patterns observed on the σC-encoding gene amplified products. Interestingly, a phylogenetic tree based on the nucleotide sequences of the σC-encoding gene also classified the Taiwanese ARV isolates into four distinct groups, indicating that the genotype was consistent with the

Table 1. RFLP profile comparison of the RT-PCR amplified product of the S1 and S4 genes of the current study with known strains of ARVs (Liu et al., 2004; Bruhn et al., 2005).

ARV Strains/isolates	Segment	TaqI	BcnI	HaeIII	HincII	DdeI
S1133 (Standard)	S1	430 - 880	1023	1023	320-620	480
	S4	350	437	120	437	340
S1133 (Vaccine).	S1	430 - 880	1023	1023	320-620	480
	S4	350	437	120	437	340
750505	S1	430 - 880	1023	1023	320-620	480
	S4	350	437	120	437	340
ARVIR1, ARVIR2, ARVIR3, ARVIR4, ARVIR5	S1	430 - 880	1023	1023	320-620	480
	S4	350	437	120	437	340
601G	S1	430-620-980-1000	1023	720	120-420-560-620	220-380-480
	S4	150	420	437	437	320
601S1	S1	430 - 880	1023	1023	320-620	480
	S4	350	80	120	437	437
R2/TW	S1	430-620-980-1000	1023	720	120-420-560-620	220-380-480
	S4	437	420	437	280	437

types based on the restriction enzyme fragment length polymorphism (Liu et al., 2004). In the present study, we also used the enzymes used by Liu et al., (2004) which were able to differentiate the ARV isolates.

For the first time in Iran, an ARV was isolated from chickens with the malabsorption syndrome and arthritis/tenosynovitis disorder, which was then characterized (Khodashenas and Aghakhan, 1992). In 2008, among the 582 serum samples obtained from broiler flocks in the Tehran province of Iran, 572 serum samples were positive for the presence of anti-ARV antibodies. In that study, the prevalence of reovirus infection was estimated to be 98.3% (Bokaie et al., 2008). Avian reoviruses have also been detected using molecular techniques in Iran (Harzandi et al., 2006). The RT-PCR and Nested PCR of the S1 gene were applied to confirm the presence of ARVs in tissue samples provided from suspicious flocks in some provinces of Iran, in which only one sample out of 28 samples was positive (Harzandi et al., 2006). The findings not only confirmed the presence of ARVs but also revealed that molecular methods could be more sensitive and even more rapid for the detection of avian reoviruses (Harzandi et al., 2006).

In this study 800 fecal swab samples were taken from breeder flocks in Iran. They were pooled and finally 120 samples were obtained for the experiment. The final samples were subjected to the RT-PCR for the S1, S4 genes of avian reovirus. After that, they were digested by five restriction enzymes, and finally compared with the standard strains. The findings showed that five samples were positive with the S1 primer and 6 samples with the S4 one. The patterns observed after the digestion of the positive PCR products revealed that the isolates of this study were identical to the S1133 and 750505 standard strains. It may be argued that since the S1133 vaccine and standard strains have identical RFLP patterns, our isolates might be vaccinal strains. However, some studies have shown that the S1133 vaccine strain would not excreted into feces (Mukiibi-Muka et al., 1984). No virus excretion in the feces was observed in two and three weeks after the vaccine administration through four different routes (i.e. drinking water, eye drop, intramascular and subcutaneous) (Mukiibi-Muka et al., 1984).

Some of our isolates were sequenced for further molecular and phylogenetic analysis. The sequencing of the isolates (data not shown) confirmed that the genotypes of the Iranian ARV isolates were consistent with types based on the RFLP of the σC-encoding and σNS-encoding genes of ARV.

The findings of this study suggested that the PCR-RFLP analysis can be considered as a simple and rapid approach for the differentiation of ARV isolates. In addition, it is a useful technique to determine whether a new variant strain is introduced into a flock or a given virus strain is spread from one flock to another.

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شناسایی رئوویروس‌های ایجادگر تنوسینوویت در پرندگان از گله‌های مرغ مادر ایران به روش RT-PCR و RFLP

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چکیده

زمینه مطالعه: رئوویروس پرندگان از مهمترین عوامل بیماری‌های پرندگان به‌ویژه آرتریت رئوویروسی، تنوسینوویت، بیماری مزمن تنفسی، و سندرم سوء جذب می‌باشند. رخداد تنوسینوویت در مرغان مادر از سایر رده های طیور پرورشی بیشتر می‌باشد. **هدف:** هدف از این بررسی شناسایی رئوویروس های ایجادگر تنوسینوویت در پرندگان از گله های مرغ مادر ایران به روش RT-PCR و RFLP بود. **روش کار:** در این مطالعه از گله های مرغ مادر باسن بالای ۴۵ هفته، ۸۰۰ نمونه مدفوعی باسواپ اخذ گردید و در محیط PBS به آزمایشگاه منتقل گردید و در نهایت بعد از مخلوط کردن نمونه ها از یک مزرعه، ۱۲۰ نمونه جهت بررسی حضور رئوویروس ها مورد استفاده قرار گرفت. در این مطالعه برای تشخیص رئوویروس های ایجادگر تنوسینوویت از پرایمرهایی اختصاصی برای نواحی ژنومی S1 با باند هدف ۱۰۲۳ pb و S4 با باند هدف ۴۳۷bp استفاده شد. **نتایج:** بعد از انجام آزمایش PCR، ۵ نمونه با استفاده از پرایمرهای ناحیه ژنومی S1 و ۶ نمونه با استفاده از پرایمرهای ناحیه ژنومی S4 مثبت شدند که موارد مثبت با ۵ آنزیم هضم کننده مورد هضم آنزیمی قرار گرفتند. آنالیز قطعات حاصله از هضم آنزیمی محصولات PCR در تمامی نمونه های مثبت دلالت بر همسان بودن الگوی هضم آنزیمی نمونه‌ها با الگوی هضم هر دو سویه واکسن و استاندارد S1133 داشت. **نتیجه‌گیری نهایی:** در این بررسی مشخص شد که بکارگیری روش مولکولی RT-PCR/RFLP روش مناسبی جهت شناسایی رئوویروس‌ها موجود در مزرعه و متمایز کردن آنها از همدیگر می‌باشد. این مطالعه اولین مطالعه مولکولی بر روی رئوویروس های ایجادگر تنوسینوویت در ایران می باشد که بر مبنای شناسایی ۲ ناحیه ژنومی S1 و S4 و نیز بکارگیری ۵ آنزیم هضم کننده در گله های مرغ مادر در ایران صورت گرفت.

واژه‌های کلیدی: رئوویروس پرندگان، گله های مرغ مادر، RT-PCR، RFLP

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