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Genotypic characterization of two novel avian orthoreoviruses isolated in Iran from broilers with viral arthritis and malabsorption syndrome

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Abstract

Background: Avian reovirus (ARV) is a major poultry pathogen associated with arthritis, malabsorption, and enteric diseases in chickens. In recent years, emerging ARV strains have become a growing concern causing significant economic losses in broiler chickens around the world. This report focuses on the isolation of ARV from the clinical occurrence of ARV-associated diseases in commercial broiler chickens in Iran and the genotypic characterization of the selected isolates. **Case description:** In 2018, two distinct clinical diseases, suggestive of malabsorption syndrome (MAS) and viral arthritis, were noticed in commercial broiler chickens in the north of Iran. Laboratory investigations were carried out following necropsy, documentation of the gross lesions, and sampling of the affected tissues for histopathology and virology. Molecular diagnosis and characterization of ARV were performed targeting Sigma C (σC) gene sequences of the virus. **Findings/treatment and outcome:** Two variant ARV strains were isolated from tendon and gizzard of broilers with clinical viral arthritis and MAS, respectively. Phylogenetic analysis of the ARV σC gene sequences revealed that field isolates were clustered in genotypes 2 and 4 (which were distinct from previous Iranian field ARV strains) with relatively low sequence identity (59.2% and 49.1%) to the classical vaccine strains (S1133 and 1733) in genotype 1. **Conclusion:** This report, for the first time, represents new emerging ARV variants associated with clinical events in Iran, providing insights on the diversity of endemic ARV field isolates, and urges the need for national-wide surveillance of ARV.

Key words: Avian reovirus, Malabsorption, Sigma C gene, Variant, Viral arthritis

Introduction

Avian reoviruses (ARVs) are members of the genus *Orthoreovirus* of the family *Reoviridae*. They are associated with a variety of pathological outcomes in commercial poultry including viral arthritis, runting-stunting syndrome (RSS), and malabsorption syndrome (MAS). However, their primary role as a causative agent has conclusively been determined for viral arthritis (Pitcovski and Goyal, 2020). The economic impact of ARV infection in commercial settings is considerable as it results in elevated mortality, poor weight gain, loss of flock uniformity, and condemnation at processing plants (Sellers, 2017). Chickens are most susceptible to ARV infection at a younger age. Hence, control of ARV infection in broilers has historically been aimed by antibodies transferred to young progenies following vaccination of the breeders with a combination of the commercial live attenuated and inactivated vaccines (Pitcovski and Goyal, 2020).

The ARV virion contains 10 double-stranded (ds) RNA genome segments (L1-L3, M1-M3, and S1-S4) (Spandidos and Graham, 1976). The segmented dsRNA genome of ARV favors mutation, recombination, and reassortment events resulting in the emergence of ARV variants that become a recent challenge to the broiler industry across the world (Bányai *et al*., 2011; Ayalew *et al*., 2017; Zhang *et al*., 2019). These variants of reoviruses were isolated from progenies of ARV-vaccinated breeders and were antigenically and genetically distinct from classical commercial vaccine strains (Troxler *et al*., 2013; Lu *et al*., 2015) suggesting insufficient protection of conventional commercial vaccines against emerging variant strains.

In the past few years, the Iranian broiler industry has experienced a rising number of viral arthritis outbreaks even with the vaccination of local breeders against ARVs (Mirbagheri *et al*., 2020). So far, published data on circulating ARVs in Iran suggests homogeneity of the field isolates grouped in genotype 1 with vaccine strains (Hedayati *et al*., 2013; Hedayati *et al*., 2016; Mayahi *et al*., 2019; Mirbagheri *et al*., 2020). In this report, we genetically characterize two novel ARV variants associated with clinical events of MAS and viral arthritis

from commercial broiler flocks in the north of Iran.

Case description

Source of the field isolates

Case 1

In February 2018, chronic feed passage, impaired weight gains, and poor feed conversion together with slightly elevated mortality (0.12-0.16% per day) were noticed in three commercial broiler flocks (10,000-15,000 chickens) under the same operation located in Golestan province, Iran. At the time of necropsy, birds were 28-day-old and exhibited various lesions in the liver and gizzard. After documentation of the gross lesions, tissue samples were collected from the liver and gizzard of the affected chickens in each of three flocks for histopathological and virological investigations and stored in 10% buffered formalin at room temperature or frozen at -20°C, respectively.

Case 2

In September 2018, signs of severe lameness were observed in birds from a 4-week-old commercial broiler flock of 20,000 chickens located in Mazandaran province, Iran. Approximately 5% of the birds showed varying degrees of lameness and splay-leg with marked lesions in the intertarsal joints region. Laboratory investigations were carried out following routine necropsy, documentation of gross lesions, and sampling of gastrocnemius tendons for virology, accordingly.

Histopathology

Sections of the gizzard and liver (from case 1) were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 3 µm using a Microm HM 360 microtome (MicromLaborgeräte GmbH, Walldorf, Germany), mounted on glass slides and stained with hematoxylin and eosin (H&E) by routine methods.

Cell culture and virus isolation

Liver and gizzard tissues from the case suspected to MAS and tendon samples from broilers suspected of viral arthritis were subjected to virus isolation in chicken embryo liver (CEL) cells (Table 1). For that, each tissue sample was separately homogenized in phosphate-buffered saline (20% w/v) containing 1 mg/ml streptomycin and 100,000 IU/ml penicillin using a T-25 digital Ultra-Turraxt (IKA, Staufen, Germany). Tissue homogenates were subjected to three freeze-thaw cycles, clarified by centrifugation, and filter-sterilized using 0.2 µm syringe filters (VWR, Vienna, Austria). 500 µL of final suspension was inoculated to primary CEL cells prepared from 14-day-old SPF chicken embryos (VALO Biomedia GmbH, Osterholz-Scharmbeck, Germany) following the protocol of Schat and Sellers (2008). The cells were incubated at 37.8°C in 5% CO_2 for 5 days or until a cytopathic effect (CPE) was observed. A sample was considered negative when no CPE was noticed after three passages.

Nucleic acid extraction, RT-PCR, and PCR

Viral RNA was extracted from 200 µL of clarified supernatant from CEL cell culture showing CPE using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. To detect and type ARV, extracted RNA was subjected to a conventional RT-PCR amplifying a 1088 bp fragment of the ARV S1 segment using the published primers P1/P4 (Kant *et al*., 2003). Furthermore, PCR for fowl adenovirus (FAdV) was carried out targeting the loop-1 region of the *hexon* gene (Meulemans *et al*., 2001) after DNA extraction from cell culture supernatant using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

RT-PCR product purification, sequencing, and phylogenetic analysis

The target 1088 bp bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sent to LGC Genomics (Berlin, Germany) for sequencing. ARV sequences obtained by sequencing were assembled using Accelrys gene, V.2.5 (Accelrys, SanDiego, USA), and analysis was performed on 981 nucleotides of the Sigma C (σC) gene located in the S1 segment. Multiple sequence alignment was performed using ClustalW and the phylogenetic tree was constructed with the Neighbour-Joining (NJ) method applying 1000 bootstrap trials in the MEGA X: molecular evolutionary genetics analysis across computing platforms (Kumar *et al*., 2018). The visualization of σC sequence alignment was performed using the mVISTA online platform (http://genome.lbl.

Table 1: Overview of the virology and PCR results

Case	Flock	Tissue	Virus isolation	RT-PCR (P1-P4[b])	PCR (HexA/B[c])
1 (MAS)	1	Gizzard	3/3[a]	3/3	0/3
		Liver	0/2	0/2	0/2
	2	Gizzard	2/2	2/2	0/2
		Liver	0/1	0/1	0/1
	3	Gizzard	2/2	2/2	0/2
		Liver	0/1	0/1	0/1
2 (viral arthritis)	-	Tendon	2/2	2/2	0/2

[a] No. samples positive/no. samples examined, [b] Conventional RT-PCR to detect ARVs according to Kant *et al*. (2003), and [c] Conventional PCR to detect FAdVs according to Meulemans *et al*. (2001)

gov/vista/mvista/submit.shtml). For the phylogenetic analysis, nucleotide sequences of reference strains representing previously described genotypic groups for ARV, sequences of commercially available vaccines, and ARV field isolates from around the world were retrieved from GenBank. The nucleotide sequence of the σC gene acquired in this report has been submitted to GenBank under accession numbers MZ520137 and MZ520138.

Results

Gross findings, histopathology, cell culture, and molecular investigations

Various lesions have been identified in the gizzard and liver of the affected broilers in the three flocks, from case 1, associated with MAS. Some birds had pale livers with small scattered white foci. The gizzard lesions included roughening, discoloration, and uneven detachment of the koilin layer. A small single area of erosion could be seen in some gizzards (Fig. 1A). No macroscopic lesions were noticed in other organs of the affected chickens. Viral arthritis-affected broilers showed unilateral or bilateral swelling of the hock joint. Removal of the skin at necropsy revealed subcutaneous hemorrhage due to partial to complete laceration of the gastrocnemius and digital flexor tendons (Fig. 1B). In some birds, clear straw-colored exudate was present around the affected tendon sheaths (Fig. 1B). No other lesions were observed in other organs especially in the digestive tract. Microscopic examination of the tissues obtained from the MAS case revealed hepatocellular degeneration and necrosis with infiltration of inflammatory cells in livers (Fig. 2A). Affected gizzards showed fragmentation of the koilin layer with the presence of necrotic cells within. Furthermore, necrosis of the granular epithelium with inflammatory cell infiltration was observed in the gizzard mucosa (Fig. 2B). During the virological examination, gizzard tissues from three MAS-affected flocks and tendon samples of viral arthritis-affected birds showed cytopathic effect

Fig. 1: Gross lesions of the gizzard and leg associated with the clinical cases of MAS and viral arthritis, respectively. (**A**) Discoloration, roughening, and detachment of the koilin layer. Single or multifocal areas of erosion could be seen in some gizzards (arrowhead), and (**B**) Swelling of the joint together with mild to severe subcutaneous hemorrhage due to partial to complete tearing of the gastrocnemius and digital flexor tendons (arrowhead). In some birds, clear straw-colored exudate was present around affected tendon sheaths (asterisk)

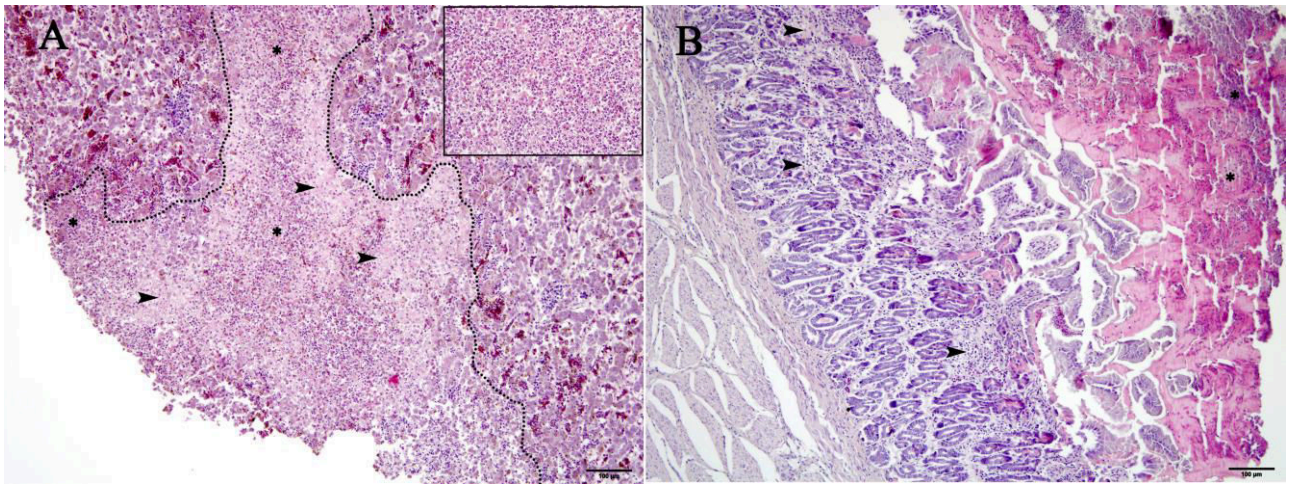

Fig. 2: Histopathology of liver and gizzard associated with the MAS case. (**A**) Multifocal areas of necrosis throughout the hepatic parenchyma (dotted line). Necrotic areas are characterized by loss and replacement of hepatocytes by pale eosinophilic remnant (arrowheads) and infiltration of inflammatory cells (asterisks). Insert: Infiltration of abundant heterophils and mononuclear inflammatory cells, (H&E, scale bar: 100 μm), and (**B**) Fragmentation of the koilin layer with necrotic materials confined within (asterisks). Degeneration and necrosis of the glandular epithelium with infiltration of the inflammatory cells (arrowheads), (H&E, scale bar: 100 μm)

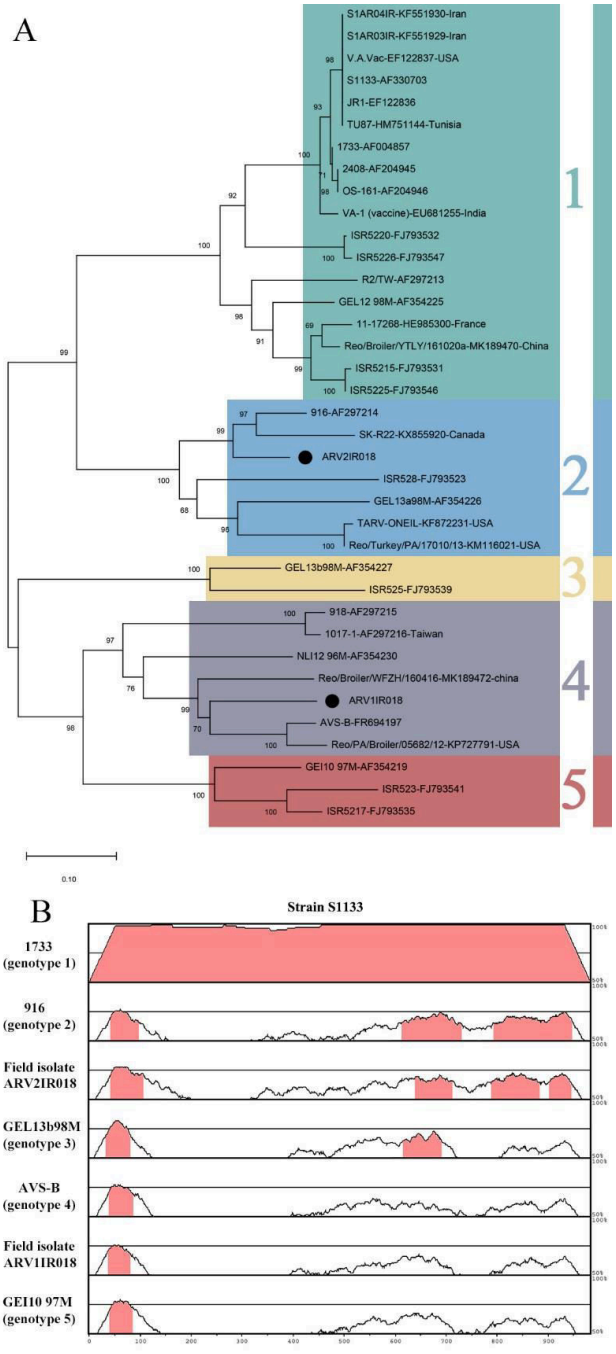

Fig. 3: Genotypic characterization of ARVs. (**A**) Phylogenetic tree based on σC protein sequences of ARVs, including the field isolate of the present report (designated by the black circles) and isolates belonging to genotype 1-5 retrieved from GenBank. ARV genotypes are labeled according to Kant *et al.* (2003), and (**B**) The mVISTA sigma C gene nucleotide alignment visualization, comparing ARV field isolates of the present study (ARV1IR018 and ARV2IR018) and reference strains representative of 5 ARV genotypes (1733, 916, GEL13b98M, AVS-B, and GEI10 97M) with strain S1133 (vaccine strain). The pink-colored areas indicate ≥70% conservation and white areas indicate <70% similarities

(CPE) compatible with ARV infection (detachment of monolayer and formation of syncytium) in cell culture; no liver samples associated with MAS-affected flocks yielded positive results (Table 1). Tendon and gizzard samples were positive within the first and third passage, respectively. Investigation of the positive cell culture supernatants with ARV-specific RT-PCR showed positive results for all samples. Neither gizzard nor tendon samples were positive for FAdV.

Genotyping clusters of the ARV field isolates

Phylogenetic analysis of the σC protein revealed that two field isolates designated ARV1IR018 (MAS isolate) and ARV2IR018 (viral arthritis isolate), clustered in the respective genotypes 4 and 2 according to Kant *et al.* (2003) (Fig. 3A). Pairwise comparison of the σC coding region showed ARV1IR018 had the highest sequence identity (80.1%) with the ARV reference strain AVS-B, which was isolated from MAS-affected broiler chickens in Delaware, USA in 2006 (Banyai *et al.*, 2011). Among three ARV reference strains (916, ISR528, GEL13A 98M) within genotype 2, ARV2IR018 had a close similarity (86.8%) to strain 916. Two variant ARV field isolates (belonging to genotypes 4 and 2) had 52.5% nucleotide homology to each other and were considerably diverse (49.1% and 59.2%, respectively) from the ARV vaccine strains (S1133, 1733, and 2408), grouped into genotype 1 (Fig. 3B).

Discussion

Currently, the poultry industry worldwide is challenged with the evolution of new ARV variants (Sellers, 2017). Although the traditional ARV live-attenuated and killed vaccines are available, field evidence shows that they do not confer protection against newly emerging variants (Troxler *et al.*, 2013). Extreme variability is the nature of ARVs; to update vaccination strategies, an un-resting characterization of field ARVs is required. Molecular classification of ARV field isolates has helped to unravel the evolution and epidemiology of emerging variant virus strains. To this end, the σC gene of ARV, the most variable region in the viral genome, has been successfully used as a genetic marker for the classification and differentiation of the field isolates. The present report focuses on the isolation and molecular characterization of two emerging ARV variants associated with MAS and viral arthritis in commercial broiler chickens in Iran.

ARVs diseases show diverse pathogenesis. Their association to clinical condition was well described in viral arthritis, but they were also isolated from chicken with MAS, RSS, hydropericardium, hepatitis, and respiratory/enteric diseases; some of them might be accidental findings awaiting experimental reproduction (Pitcovski and Goyal, 2020). Accordingly, signs and lesions of viral arthritis-affected broilers in the present report were comparable to the characteristics described for the disease (Pitcovski and Goyal, 2020). The pathogenesis of the MAS is not well understood. The condition is generally characterized by lowered body weights due to impaired digestion linked to several possible agents including ARV. Similar to the present report, hepatitis and occasionally gizzard lesions have

been documented in broiler chickens with ARV-associated gastrointestinal disease (Lenz *et al*., 1998). Regarding gizzard's lesion, an early study on gastrointestinal pathogenicity of ARV and FAdV field isolates in specific-pathogen-free chickens revealed that ARV could induce mild erosions in gizzards while FAdV caused marked gizzard lesions (Lenz *et al*., 1998). However, it is now recognized that FAdV rather than ARV plays an important role in the pathogenesis of gizzard erosion in chickens (Schachner *et al*., 2018). Overall, to explain the gastrointestinal pathogenicity of the field isolate, particularly the lesions in the gizzard, experimental confirmation is required.

Phylogenetic analysis of the σC protein showed the separation of two field isolates from earlier Iranian ARV field isolates and commercial vaccine strains within genotype 1. The findings of this report suggest a higher diversity of the Iranian ARVs; one isolate (designated as ARV2IR018), fell into a distinct subgroup within genotype 2 with 86.8% amino acid sequences similarity to the closest ARV reference strain within the same genotype (916) and 59.2% identity to commercial vaccine strains in genotype 1. Genotype 2 ARVs have recently been isolated from broilers with tenosynovitis in the USA, Canada, and China (Lu *et al*., 2015; Ayalew *et al*., 2017; Chen *et al*., 2020; Yan *et al*., 2021) confirming the widespread distribution of this genotype. The second field strain (designated as ARV1IR018), isolated from MAS case, clustered into genotype 4 and was <50% similar to commercial vaccine strains; the isolate was 80.1% similar to the reference strain AVS-B, which was also a MAS ARVs (Banyai *et al*., 2011). However, in recent years, an increased number of ARV field isolates with the close phylogenetic relationship with AVS-B have been reported also in viral arthritis-affected broilers from around the world (Lu *et al*., 2015; Chen *et al*., 2020, Egaña-Labrin *et al*., 2021; Mase *et al*., 2021) suggesting that reassortment occurs between MAS ARVs and viral arthritis ARVs.

Protection against ARVs was found to be type-specific (Wickramasinghe *et al*., 1993). Hence, to achieve competent protection, neutralizing antibodies must be raised against the prevalent ARV genotypes that exist in the field (Lublin *et al*., 2011). As a suitable preventive measure, autogenous vaccines, manufactured with local pathogenic ARV field isolates, in addition to the commercially available vaccines are practiced in regions with intensive broiler production (Sellers *et al*., 2017). The inclusion of local ARV isolates in an effective autogenous vaccine requires adequate field and laboratory data on the prevalence of clinically important ARVs within a region. Results of the present report raise concerns about the presence of clinically relevant variant ARV isolates in Iran and provide the rationale for national-wide surveillance of ARVs.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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