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Genotyping of avian infectious bronchitis virus in Iran: Detection of D274 and changing in the genotypes rate

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ABSTRACT

The coronavirus avian Infectious bronchitis virus (IBV) poses economic threats to poultry farms worldwide, affecting the performance of both meat-type and egg-laying birds. To define the evolution of recent IBVs in Iran, a genetic analysis based on hypervariable nucleotide sequences of S1 gene was carried out. Tracheal swab samples were collected from 170 Broiler flocks during 2017. Ten tracheal swabs from each flock pooled. From a total number of 170 flocks tested, 84.71% found to be positive. Phylogenetic tree analysis revealed the presence of D274 as a first time in Iran. IS/1494/06 was showed to be dominant IBV type circulating in broiler farms with a significantly higher prevalence than other four genotypes. Considering fluctuations in QX-type prevalence in recent years, continuous monitoring is necessary to reduce economic consequences in layer and broiler farms. The findings highlight the importance of using modified vaccination strategies that are adapted to the changing disease scenario.

1. Introduction

Avian Infectious bronchitis virus is the prototype *gammacoronavirus* in the family *Coronaviridae*, order *Nidovirales*. As its name implies Avian infectious bronchitis virus (IBV) is responsible for acute respiratory tract infections in chickens. Laying hens experience reduced production, eggshell abnormalities, and decreased internal egg quality. In addition to tracheal damage, some strains of IBV can cause renal lesions. The positive-sense viral genome 27.6 kb in length encodes at least ten open reading frames (ORFs) organized as follows: 5′ UTR-1a-1ab-S-3a-3b-E-M-5a-5b-N-3a-3′UTR. ORFs 1a and 1b encode 15 non-structural proteins responsible for viral replication and pathogenesis. Four structural proteins including the spike glycoprotein (S), small membrane protein (E), membrane glycoprotein (M), and nucleocapsid protein (N) are encoded by mRNAs 2, 3, 4 and 6, respectively [1,2]. The spike protein is post-translationally cleaved into S1 and S2. The S1 subunit is the main target of neutralizing antibodies. Evolution in IBV is essentially associated with the sequences of the S1 glycoprotein, and the genetic diversity of IBV is principally screened by analysis of the S1 gene. Mutations in the S1 leads to the emergence of variant serotypes which do not confer complete cross-protection against each other [3–6]. Among a large number of serotypes or variants emerged after the first documentation of IBV in 1931, Massachusetts 41 (M41) and 793/B serotypes have been expanded through the world and commercial vaccines are available against both serotypes providing a broad cross-protection against many different IBV types when used together [7–9]. Despite vaccination efforts, novel field IBVs continue to emerge; some antigenic variants may subsequently become dominant which makes prevention of IBV infections very challenging [10,11]. Similar to what has been happening in Iranian poultry farms leading to unceasing IB outbreaks despite extensive vaccination. In this work, we focus on potential IBV variants escaping from vaccine-induced immunity.

2. Material and methods

2.1. Sample collection

Tracheal swab samples were collected from 170 broiler flocks representing signs of the respiratory complex in 2017. Chickens had a history of vaccination against IBV (Massachusetts + 793/B type

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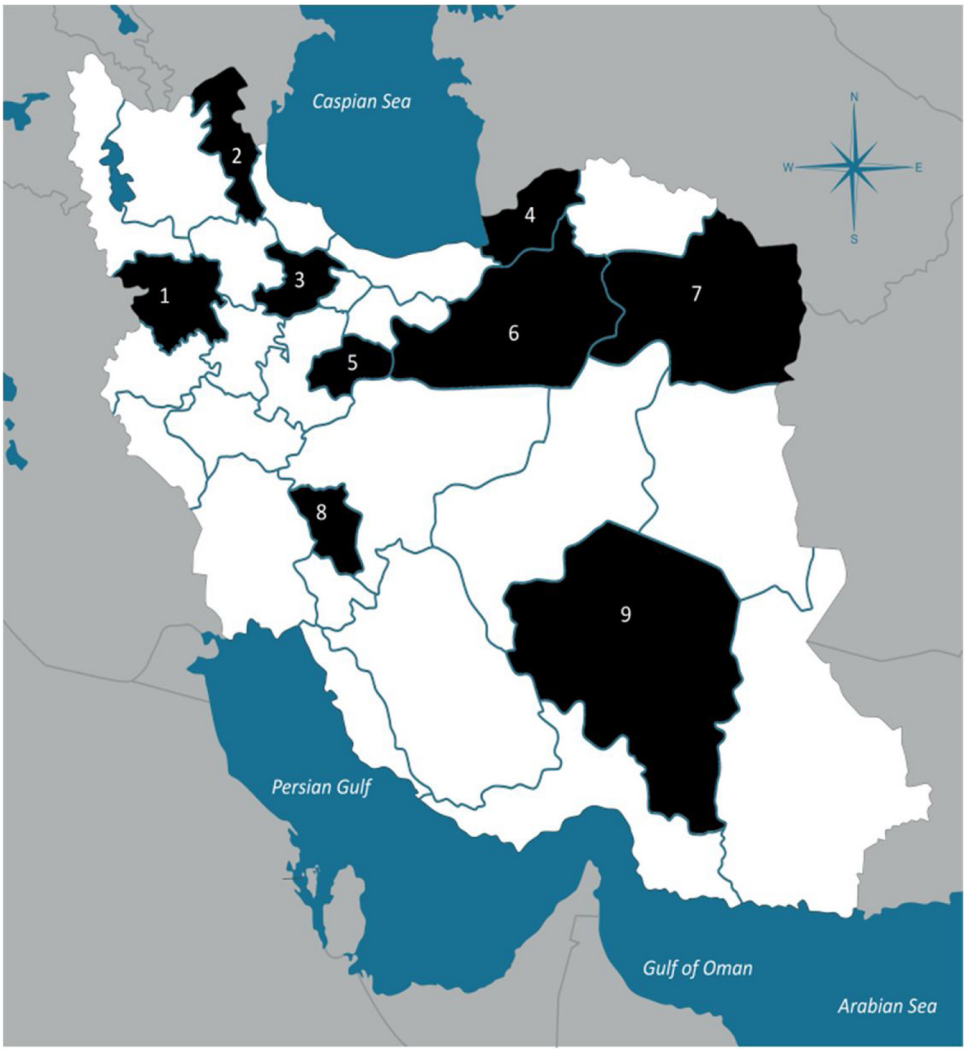

Fig. 1. Geographical locations of provinces selected for sample collection.
1, Kurdistan; 2, Ardabil; 3, Qazvin; 4, Golestan; 5, Qom; 6, Semnan; 7, Khurasan Razavi; 8, Chahrmahal-E-Bakhtiari; 9, Kerman.

vaccines). The chicken farms were located in nine different provinces of Iran including Qazvin, Ardabil, Semnan, Kerman, Khurasan Razavi, Chahrmahal-E-Bakhtiari, Kurdistan, Qom, and Golestan. Geographical locations of these provinces are shown in Fig. 1.

2.2. RNA extraction and cDNA synthesis

Ten tracheal swabs from each flock were pooled. Homogenized pooled samples were submitted for RNA isolation using RNA easy mini kit (Qiagen). The cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) [12].

2.3. Real-time PCR for IBV detection and RT-PCR for IBV Genotyping

A previously designated Real-time PCR [13] was used for IBV detection targeting 5′ UTR of the IBV genome. IBV positive samples were submitted to a nested PCR amplification with the aim of genotyping [12]. PCR products were purified using the AccuPrep® PCR purification Kit (Bioneer Co., Korea) and submitted for sequencing (Bioneer Co., Korea).

The AccuPrep® PCR purification Kit (Bioneer Co., Korea) was used for purification of the PCR products. Sequencing was performed using ABI 3100 Genetic Analyzer (Applied Biosystems, USA) with the primers (Both directions) used in the second step of nested PCR (Bioneer Co., Korea). Chromatograms were evaluated with CromasPro (CromasPro Version 1.5). The nucleotide sequences of S1 gene from Afghan IBV strains obtained in this study were subjected to BLAST (primary genotyping and similarity results), aligned and compared with reference strains downloaded from NCBI GenBank database from foreign Countries and neighboring States. We remove the similar sequences Sequence homology analysis was performed using MEGA7.0. Phylogenetic trees were constructed using MEGA7.0 with the neighbor-joining algorithm (bootstrap values of 1000) with the *Kimura2* parameter model [14]. The nucleotide and amino acid sequences determined in this study are available in the GenBank under accession number: MH106448-MH106510.

3. Results

From a total number of 170 flocks tested, 84.71% found to be positive. The phylogenetic analysis revealed detected IBVs were divided into five major clusters (Fig. 2). The distribution of prevalence rates in each province is depicted in Table 1. IS-1494-like IBV was the most common type accounted for 85% of detected strains. The other types overall created a lower proportion; including 793/B having a prevalence of 7%, QX, and Mass with prevalence rates of 5% and 2%, respectively, and D274 with the incidence of 1% (Fig. 3)

The sequence identity among IS/1494/06 IBV types detected in this

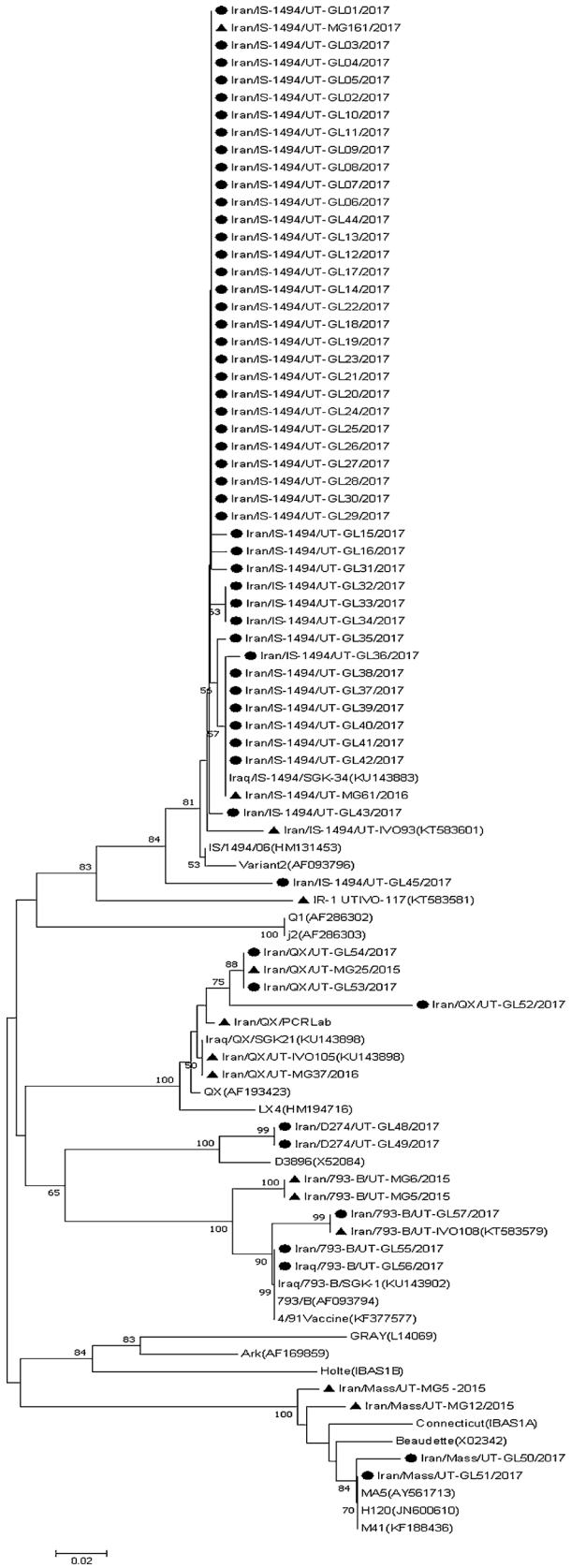

Fig. 2. Neighbor-joining phylogenetic tree based on partial S1 gene sequences of Iranian IBVs and selected reference strains. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches IBVs obtained in this study are marked with black squares; other Iranian IBVs detected in recent years are shown by black diamonds.

Table 1
Geographical distribution of IBV strains in different provinces of Iran during 2017.

Province	Positive (%)
Qazvin	93.33
Ardebil	88.00
Semnan	85.00
Kerman	85.00
Khorasan-e-Razavi	82.35
Chahrmahal-E-Bakhtiari	82.35
Qom	82.35
Golestan	82.35
Kurdestan	81.82

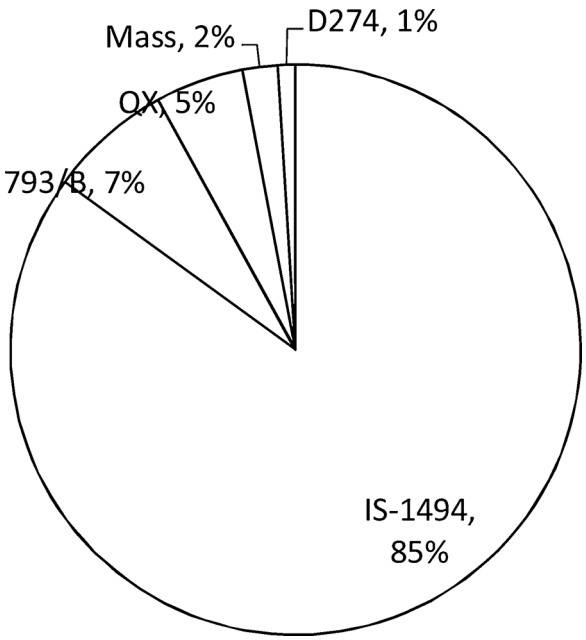

Fig. 3. Proportion of each IBV type circulating in Iran from 2017.

work and reference strains was more than 98.86%. Mass IBVs shared more than 98.28% sequence similarities including some strains completely identical to the H120 vaccine. 793/B type IBVs in this study shared 93.29-100% sequence homology with each other, while less with those detected in the last few years. Sequence identities among QX IBVs varied between 89 to 100% (Table 2). Figs. 4 and 5 shows comparisons of partial S1 sequences of IBV strains detected in this work with some selected IBV types with 793/B and Mass type respectively (Figs. 4 and 5).

4. Discussion

Despite vaccination efforts, novel field IBVs continue to emerge in many parts of the world, leading to the dominance of some antigenic variants that makes prevention of IBV infections very challenging [10,15]. Two independent studies have been dedicated to investigating the IBV epidemiology in Iran. The first one was carried out by Najafi et al. between 2014 and 2015[16], the second was done during 2015–17 [12]. Comparison of their results with the result obtained in this study showed the growing trend in IS/1494/06 IBV prevalence (Fig.6).

The circulation of such a high rate of IS/1494/06 genotype unrelated to the used vaccine strains declaring they originated from several sources. It is one of the most prevalent IBV genotypes in Middle East countries such as Kuwait, Lebanon, Oman, Saudi Arabia [17]. High sequence similarities among IS/1494/06 IBVs in Iran and those

Table 2
Nucleotide homology between representatives from each distinct cluster and selected reference strains for partial S1 sequences.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	Iran/IS-1494/UT-GL29/2017																										
2	Iran/IS-1494/UT-GL30/2017	100																									
3	Iran/IS-1494/UT-GL28/2017	100	100																								
4	Iran/IS-1494/UT-GL22/2017	100	100	100																							
5	Iran/IS-1494/UT-GL37/2017	99.425	99.43	99.425	99.425																						
6	Iran/Mass/UT-GL50/2017	77.011	77.01	77.011	77.011	77																					
7	Iran/Mass/UT-GL51/2017	78.736	78.74	78.736	78.736	78.2	98.3																				
8	Iran/D274/UT-GL48/2017	82.184	82.18	82.184	82.184	81.6	75.3	77.01																			
9	Iran/793-B/UT-GL55/2017	81.034	81.03	81.034	81.034	80.5	69.5	71.26	81																		
10	Iran/QX/UT-GL53/2017	82.184	82.18	82.184	82.184	81.6	73.6	75.29	78.7	82.184																	
11	Iran/QX/UT-GL54/2017	82.184	82.18	82.184	82.184	81.6	73.6	75.29	78.7	82.184	100																
12	Iran/QX/UT-GL52/2017	75.862	75.86	75.862	75.862	75.3	67.2	68.97	71.8	74.138	91.38	91.4															
13	Iran/793-B/UT-GL57/2017	78.736	78.74	78.736	78.736	78.2	68.4	70.11	81	97.701	79.89	79.9	71.8														
14	Iraq/QX/SGK21(KU143898)	83.908	83.91	83.908	83.908	83.3	74.7	76.44	79.9	82.759	98.28	98.3	89.7	80.46													
15	Iran/793-B/UT-IVO108(KT583579)	78.736	78.74	78.736	78.736	78.2	68.4	70.11	81	97.701	79.89	79.9	71.8	100	80.46												
16	IR-1_UTIVO-117(KT583581)	86.207	86.21	86.207	86.207	86.8	71.3	72.41	77	78.161	79.89	79.9	72.4	75.86	81.61	75.9											
17	Iran/IS-1494/UT-MG161/2017	100	100	100	100	99.4	77	78.74	82.2	81.034	82.18	82.2	75.9	78.74	83.91	78.7	86.2										
18	Iran/793-B/UT-MG5/2015	80.46	80.46	80.46	80.46	79.9	70.1	71.84	82.2	95.977	82.18	82.2	74.1	94.83	82.76	94.8	78.2	80.5									
19	Iran/QX/UT-MG37/2016	83.908	83.91	83.908	83.908	83.3	74.7	76.44	79.9	82.759	98.28	98.3	89.7	80.46	100	80.5	81.6	83.9	82.8								
20	Iran/Mass/UT-MG5_-2015	80.46	80.46	80.46	80.46	79.9	96.6	98.28	78.7	72.989	75.86	75.9	69.5	71.84	77.01	71.8	74.1	80.5	73.6	77							
21	4/91Vaccine(KF377577)	81.034	81.03	81.034	81.034	80.5	69.5	71.26	81	100	82.18	82.2	74.1	97.7	82.76	97.7	78.2	81	96	82.8	73						
22	H120(JN600610)	78.736	78.74	78.736	78.736	78.2	98.3	100	77	71.264	75.29	75.3	69	70.11	76.44	70.1	72.4	78.7	71.8	76.4	98.3	71.3					
23	D3896(X52084)	82.759	82.76	82.759	82.759	82.2	75.3	77.01	95.4	79.885	79.31	79.3	74.1	79.89	80.46	79.9	78.7	82.8	80.5	80.5	78.7	79.9	77				
24	IS/1494/06(HM131453)	99.425	99.43	99.425	99.425	98.9	77	78.74	82.8	81.609	82.18	82.2	75.9	79.31	83.91	79.3	86.2	99.4	81	83.9	80.5	81.6	78.7	82.8			
25	QX(AF193423)	83.908	83.91	83.908	83.908	83.3	74.7	76.44	80.5	83.333	97.7	97.7	89.1	81.03	99.43	81	81.6	83.9	83.3	99.4	77	83.3	76.4	81	83.9		
26	Q1(AF286302)	81.609	81.61	81.609	81.609	81	72.4	74.14	79.3	79.31	78.16	78.2	73	77.01	79.31	77	79.9	81.6	79.3	79.3	75.9	79.3	74.1	79.9	82.2	79.9	

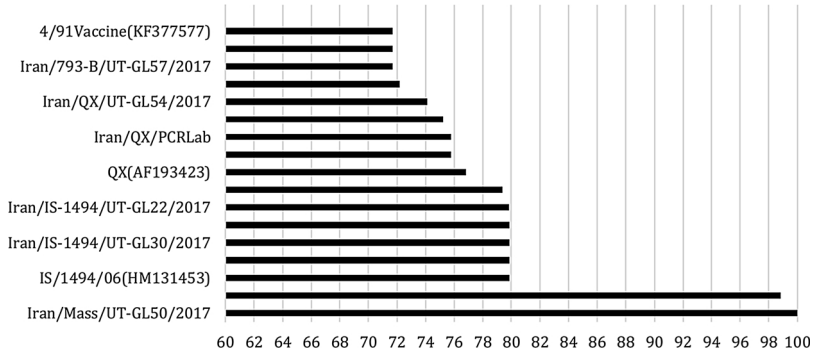

Fig. 4. Percent identity of partial S1 gene sequences of some IBVs from the current study to H120 vaccine strain.

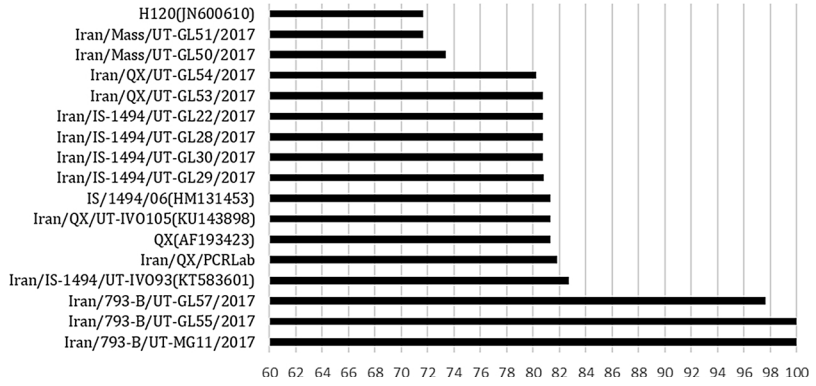

Fig. 5. Percent identity of partial S1 gene sequences of some IBVs from the current study to 793/B vaccine strain.

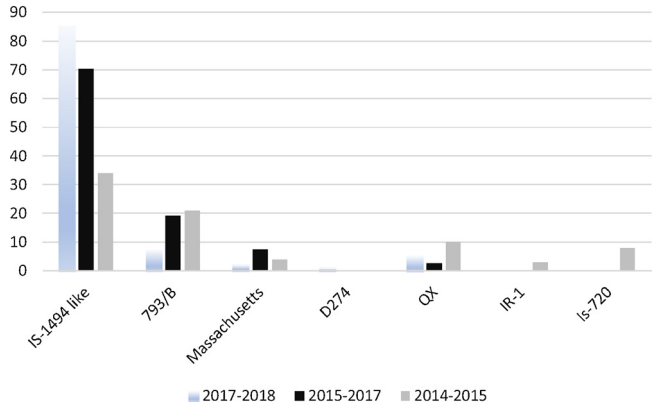

Fig. 6. Prevalence of different types IBV.

circulating in our neighboring countries including Israel, Iraq, Turkey [16,18], may explain the virus origin. It is not yet known how IS/1494/06 spread among Middle East countries; one may suspect to the role of wild birds in virus transmission [19], another possible reason is intense trade among Middle East countries and illegal movement of animals across borders. With much lower prevalence, 793/B was the second most predominant IBV type in this study. The 793/B serotype is one of IBV types used in our national vaccination program. As the method used in the present study could not differentiate field and vaccine strains, 793/B IBV type was expected to the 793/B vaccine strain use (Fig. 6). In breeder and laying hens QX-type causes delayed the onset of production, poor peak in egg production, a high percentage of false-layers and poor quality of eggs [20]. The QX prevalence obtained in the present study was 5% which could reflect the efficacy of vaccination [21]. Although the prevalence is relatively low, the 2 to 10% fluctuation in its prevalence within a few last years and also increase the genetic distance between field and vaccine strains are worrying, since it is probably currently the IB variant of most concern for breeder/ layer flocks. At the stage that the QX live vaccines are not registered in Iran.

D274, a Dutch strain was first isolated in 1979, reported to be serologically related to both A and B serotypes [22,23]. Strains D207 and D3896 considered belonging to the same serotype as D274 [24]. D207 was isolated in samples collected between 1981 and 1983 in Britain [25]. D274 isolates were recovered from vaccinated and especially non-vaccinated broilers over a 10-year period from 1986 to 1995 in Belgium [26]. It was identified in Egypt (Egypt/D274/D/89) [27]. D274 was detected in Sweden, England and Russia in 1995, 1996 and 1998, respectively [28]. Several reports have been confirmed D274 presence in Western Europe from 2002 to 2006 [19,29,30]. D274 was not detected in the Middle East countries before 2005, its first report from Jordan [31]. After that in a six-year investigation of the dynamics of IBV, D274 was detected in Oman, Saudi Arabia, and the United Arab Emirates, regarding the sequencing data, 62.50% of them identified as field and not vaccine strain[17]. D274 has been detected once in Iran using serological assays (personal communication); the present study reports the first molecular detection of D274 genotype. Since D274 is not included in any vaccine strains used in Iran, it is probably originated from neighboring countries. In addition to biosecurity practices, vaccination is usually needed to control IB. Selecting the proper vaccination schedule will be more complicated considering the existence of many IBV variants. It has been shown that vaccination with two antigenically distinct live-attenuated vaccines such as Mass and 793/B can result in a broad cross-protection against many different IBV types [15]. Awad et al. showed that administering combined live H120 and CR88 vaccines simultaneously at day-old followed by the CR88 vaccine at 14 d-old gave more than 94% tracheal ciliary protection IS/1494/06. The other vaccination program, H120 at day-old followed by CR88 at 14 d-old, the tracheal ciliary protection conferred was 80 percent from IS/1494/06-like [32]. Our experiment in which combination of H120 and 793/B provided better protection than using homologous H120-H120 vaccination strategy, while it was not still completely protect against

IS/1494/06 challenge [33].

Awad et al. showed that one-day-old chicks vaccinated with two different mixtures of Mass + 793/B produced 92% and 68% ciliary protection against QX challenge [11]. Mohammadi et al. showed a protective ability of the used vaccination program. The H120 at first day of age followed by 793/B at 14 d of age obtained 81% protection against QX variants circulating in Iran [34].

Ignoring some differences, Mass, and 793/B vaccination strategy seems more relevant in our conditions, in which we are not allowed to use D274, QX, and IS/1494/06 variant vaccines. Another challenging issue is about the application route and timing of vaccination. Vaccines are applied with spray route in a hatchery or the first day of age. Although it is preferred to apply the booster in the form of a spray, mass application through the water is often chosen as chicks grow. The main disadvantages of the latter route are inconsistencies of vaccine dosage depending on water consumption, and the potential for some birds to receive no vaccine at all. This study suggests a vaccination schedule using an initial combination of Mass + 793/B vaccine followed by a 793/B vaccine two weeks later, which appears to confer more protection than an only Mass type vaccine boosted by a 793/B vaccine and the continuing IBV surveillance necessary to make new approaches. Using the generated epidemiological data to help adjust the IB vaccination programs.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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