A. MASOUDIAN, N. SHEIKHI, M. H. BOZORGMEHRI-FARD

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Identification of new avian Infectious Bronchitis virus variants in Iranian poultry flocks by High Resolution Melting curve analysis

Masoudian A.[1], Sheikhi N.[2*], Bozorgmehri-fard M. H.[2]

[1] *PhD Student of Poultry Diseases, Department of Poultry Diseases, Faculty of Veterinary Science, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2] *Department of Poultry Diseases, Faculty of Veterinary Science, Science and Research Branch, Islamic Azad University, Tehran, Iran*

ABSTRACT. Avian Infectious bronchitis (IB) is a common coronavirus infection of chickens and responsible for performance reduction and increasing mortality due to subsequent respiratory, renal and/or reproductive disorders. Classification of causative agent is necessary to plan successful vaccination strategies to prevent the infection due to poor inter-strains cross-reaction. To identify dominant circulating strains in Iran, a Real-time PCR combined with 3' Un-Translated Region (3' UTR) High Resolution Melting (HRM) analysis designed as a rapid and reliable method for IB Virus (IBV) detection and differentiation. Samples collected from 20-suspected flocks and after PCR products, HRM curves of samples as well as 6 commercial IB live vaccines with 2 standard strains, were analyzed as references. IBV genomes detected in 11 samples while according to HRM analysis and calculating Genotype Confidence Percentage (GCP), 6 positive specimens identified as 793/B field strains and the left 5 found as new IBV variant strains. Then obtained PCR products sent for nucleotide sequencing to determine genotype relativity. All five infectious agents, related to QX-like type and indicating circulation of new variants in Iran as a probable cause of vaccination failures and consequent economical losses.

Key words: High Resolution Melting analysis, Identification, Infectious Bronchitis Virus, Iranian, New variant, Poultry

Corresponding Author:
Sheikhi N., Science and Research Branch of Islamic Azad University, Hesarak Boulevard, University Square, The end of Satari martyr highway, Tehran, Iran.
Postal code: 14515/775
Email: sheikhinariman@gmail.com

1. INTRODUCTION

Avian Infectious Bronchitis Virus (IBV) considered as a causative agent of a worldwide, acute, highly contagious disease of chicken called (IB). IBV belongs to group 3 coronavirus genus in Coronaviridae family. Its genome consists of 27,000 bp nucleotides coding four structural proteins including Spike (S), Membrane (M), Nucleocapsid (N) and envelope (E) (Cavanagh and Gelb, 2008). In susceptible chicks, IBV causes respiratory disease followed by reduced weight gain, increased feed conversion ratio (FCR) and mortality, especially by concurrent bacterial infections. In commercial layers and breeders, IBV replication in oviduct causes decrease in egg production and quality. IBV is highly transmissible and the only way to control it is by prevention, because there is no treatment for IB. Although strict biosecurity measures and vaccination schedules are implemented widely, the success may not be achieved, since there is low cross-immunity between multiple serotypes of IBV and there is risk of vaccine reversion to more virulent strains. Therefore, identification of dominant IBV strains in the field is so important to avoid vaccination failures and control consequent economic losses (Cavanagh and Gelb, 2008; Cook, 2012).

Although there are several methods to differentiate and classify IBV strains, the most effective way is the nucleotide sequencing of IBV's genome, especially S1 gene. However, due to high propensity of this gene to mutation or recombination, it cannot solely be reliable (Hewson, 2009; Hewson, 2010). Moreover, S1 gene sequencing is not rapid enough to plan an appropriate vaccination protocol in order to control IBV outbreaks (Hewson, 2009; Hewson, 2010). Due to presence of hyper-variable region in the 3' UTR of IBV with conserved flanking regions, sequence analyzing of 3' UTR can be used to differentiate IBV strains (Williams, 1993)1993. It has been recognized that a deletion in the 3'UTR of the Australian subtype B vaccines that not observed in the same serotype of field isolates (Hewson, 2009). In another study suggested that the 3'UTR could be used to differentiate vaccine and field isolates by using real-time polymerase chain reaction (RT-PCR), which is more rapid than sequence analysis (Hewson, 2010).

High Resolution Melt (HRM) analysis method was reported to accurately identify single base changes in nucleotide sequences of up to 400 base pair (bp) in length (Lin, 2008).

Recently, despite vaccination, there are several reports of IB outbreaks in Iranian broiler and layer flocks. The aim of this study was to evaluate IBV strains status and determinate new variants in Iranian poultry flocks by using HRM curve analysis of IBV-3' UTR region as a rapid and reliable method for differentiation and characterization of IBV strains.

MATERIALS AND METHODS

Field sampling

Twenty tracheal samples were collected from suspected broiler and layer flocks as well as one Iranian IBV field isolate registered as IR/773/2001 (793/B) (Razi institute; Iran), one standard M41 strain (Veterinary Laboratories Agency Central Veterinary Laboratory, Weybridge; UK) and seven current IB commercial live vaccines, as reference strains (Table1).

Table 1: IBV strains

| References	Specification	Sources
Vac.1	Nobilis IB 4/91(793/B)	Merck Animal Health-Netherland
Vac.2	Nobilis Ma5	Merck Animal Health- Netherland
Vac.3	Bronhikal SPF (H120)	Veterina- Croatia
Vac.4	H120	Razi- Iran
Vac.5	Cevac Vitabron L (H120+PHY.LMV.42)	Ceva- Hungary
Vac.6	Bioral H120	Merial- France
Ref.1	M41	Veterinary Laboratories Agency, Weybridge-UK
Ref.2	IR/773/2001 (793/B)	Razi-Iran

VIRAL RNA EXTRACTION AND CDNA SYNTHESIS

Easy-spin® RNA extraction kit (iNtron Biotechnology inc; South Korea) was applied to extract viral RNA. The tracheal scrapings were homogenized in PBS and all vaccines and other reference strains were diluted to 2 ml distilled water before RNA extraction. To start RNA extraction, 100 μl of diluted vaccine or PBS containing field tracheal scrapings, lysed in 900 μl of Lysis buffer and continued according to the manufacturer's instruction. The extracted RNA was added to a reaction micro-tube contains 5 μl final solution including 20 units Ribolock TM (RNase inhibitor) enzyme, 10 mM dNTPs, 200 units RevertAid TM (murine Moloney leukemia virus reverse transcriptase), 100 pmol Randome Hexamer, 12.5 μl DEPC- treated water (Fermentas- Termo Fisher Scientific; USA). This was incubated at 25 degree centigrade (ºC) for 10 minutes, after which it was heated at 42 ºC for 60 minutes and then 70 ºC for 70 minutes (Hewson, 2009; Hewson, 2010).

Real-time RT-PCR and HRM analysis

For Real-time RT-PCR, all of 25 μl RT reactions contained 2.5 μl 10× Buffer, 5 μM Syto9® (Invitrogen), 50 mM MgCl2, 1.2 μM dNTP, 10 mM forward primer and 10 mM reverse primer, 2 μl cDNA as template. The sequence of the forward primer (All 1-F) was CAGCGCCAAAACAACAGCG and reverse primer (Del 1-R) was CATTTCCCTGGCGATAGAC (Cinagen; Iran) (Hewson, 2009; Hewson, 2010). The 3' UTR nucleotide sequences for vaccine strains and references were unavailable in GenBank.

RT-PCR was performed using a Rotorgene 6000 (Corbett Life Science; Germany) with an initial denaturation step of 94 ºC for 1 minute, followed by 40 cycles of 94 ºC for 20 seconds, 57 ºC for 20 seconds, 72 ºC for 30 seconds, and a final extension of 72 ºC for 2 minutes (Hewson, 2009; Hewson, 2010).

HRM curve analysis was carried out on the 3' UTR PCR products immediately after PCR using a Rotorgene 6000 and Rotor-Gene Q Series 2.0.2.4 version (Corbett Life Science). The 3' UTR PCR prod-
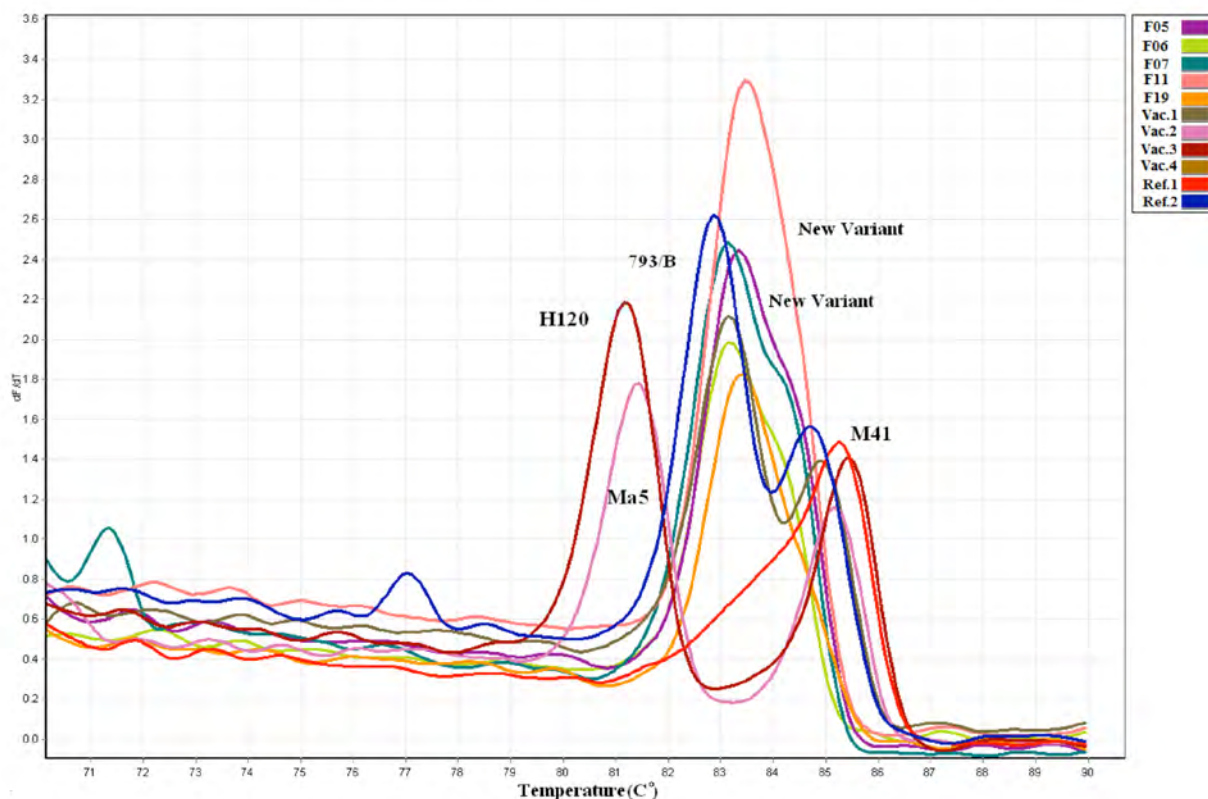
Fig 1: Conventional melt curves produced by the amplicons from 3' UTR of F.05, F06, F.07, F. 11 and F.19 field samples, determined as Varaint' QX-like type and compated to refrence strains and vaccines.

ucts were subjected to temperature increasing ramps of 0.3 ºC between 70 ºC and 90 ºC (Hewson, 2009; Hewson, 2010).

Then each strain was set as a 'genotype' comparing to known reference strains and the HRM Genotype Confidence Percentages (GCPs), valued attributed to each strain being compared to each genotype. Strains with GCP equivalent or more than 95% indicating the same genotype.

After HRM analysis of positive field samples, amplicons related to the new variants sent to Kavoosh Fanavar Kosar Company to be sequenced. Then initial identification all PCR products were carried out online by BLAST software (http://blast.ncbi.nlm.nih.gov). Moreover, in order to evaluate 3'UTR sequence of the vaccine and field IB strains for planning a differentiation tool, another commercial 793/B IB vaccine (Gallivac IB88; Merial) as well as Vac.1 (Nobilis 4/91; MSD) and one field strain (IR/773/2001-Razi) were subjected to find probable differences.

Table 2: Genotype Confidence Percentage of 3' UTR amplicons obtained from 11 positive samples, refrence strains and vaccines at a ramp of 0.3 Cº

	Vac.1	Vac.2	Vac.3	Vac.4	Vac.5	Vac.6	Ref.1	Ref.2	F.01	F.02	F.03	F.04	F.05	F.06	F.07	F.11	F.12	F.19	F.20
Vac.1	100	0.18	0.09	0.08	0.07	0.07	0.05	90.58	89.97	89.80	88.65	89.02	45.20	38.17	49.18	52.20	87.44	41.69	90.89
Vac.2		100	80.87	82.43	87.40	83.34	0.00	0.48	0.44	0.15	0.38	0.07	0.03	0.05	0.05	0.02	0.78	0.03	0.04
Vac.3			100	90.00	89.26	95.77	0.01	0.20	0.15	0.04	0.17	0.03	0.01	0.01	0.01	0.23	0.01	0.03	0.09
Vac.4				100	97.37	88.50	0.00	0.23	0.19	0.06	0.18	0.03	0.01	0.02	0.02	0.01	0.36	0.01	0.02
Vac.5					100	92.05	0.00	0.25	0.15	0.05	0.12	0.01	0.01	0.01	0.01	0.08	0.21	0.02	0.00
Vac.6						100	0.01	0.15	0.10	0.03	0.13	0.02	0.01	0.01	0.01	0.00	0.16	0.01	0.03
Ref.1							100	0.01	0.12	0.20	0.07	0.93	0.81	0.40	0.05	0.10	0.33	0.03	0.37

	Ref.2	F.01	F.02	F.03	F.04	F.05	F.06	F.07	F.11	F.12	F.19	F.20
Ref.2	100	96.83	95.80	90.41	91.37	49.57	50.05	59.79	40.49	90.08	50.06	92.96
F.01		100	95.69	90.77	89.14	57.97	70.19	77.22	49.45	88.03	89.17	91.36
F.02			100	91.61	90.79	41.25	47.55	59.72	53.69	88.69	89.16	90.47
F.03				100	96.43	61.20	59.79	58.95	60.05	90.38	50.07	90.09
F.04					100	55.77	36.98	49.10	53.28	89.52	30.07	91.33
F.05						100	95.26	96.02	90.95	24.91	91.64	20.16
F.06							100	97.19	91.28	49.00	89.97	5.51
F.07								100	90.42	49.38	89.35	8.27
F.11									100	20.11	97.06	19.62
F.12										100	25.33	72.10
F.19											100	20.38
F.20												100

RESULTS

Real time RT-PCR showed 11 IBV field samples were positive. Melt curves for references have been categorized in three types including H120 and Ma5 vaccines, 793/B vaccine (Nobilis 4/91) and Razi filed isolate, M41 strain and field samples. Massachusetts strain vaccines melt curves showed two peaks in the rage of 79 to 87º C, which the first peak was taller than the second (Figure). IBV 793/B types (Vac.1 and field), had two distinct but similar melt patterns which the melt curves ranged from 81 to 86.2º C with two followed peaks that the first was taller than the second one with shorter interval as compared with Massachusetts types (Figure). Moreover, 793/B vaccinal strain (Vac.1-4/91) showed first peak at higher temperature compared to Razi field isolate (Ref.2) (Figure). Ref.1 sample (M41) generated a different melt pattern in the range of 81to 86.8 º C having just one peak (Figure). None of field samples melt curves was similar to those vaccines and reference strains. After HRM curves analysis, GCP calculated by the mentioned software and the results showed a correlation with the melt patterns of specimens (Table 2).

According to visual examination of HRM graph's patterns and comparison of GCPs, positive samples classified in six groups (Table3). Group 1, 2, 5 and 6 had similar melt curve pattern to 793/B types but just differed from Razi field isolate. Two left groups had different melt curves pattern from references and 793/B type, therefore determined as new variant types. Group 3's (F.05, F.06 and F.07) melt curves had a sharp spike-like peak at 83.5º C and group 4 (F.11 and F.19) showed a high peak with a mild bulging step in descending curve side and melt occurred between 81 to 85.2º C (Figure).

According to BLAST results, all new variants (5 filed samples) showed maximum similarity of 95% to three QX-like strains including CK/CH/LZJ/111113 (JX195175.1), CK/SWE/0658946/10 (JQ088078.1) and ITA/90254/2005(FN430414.1).

After comparing 3' UTR fragment sequence of 793/B vaccine and field strains, an 18 bp deletion found in the initial part of vaccine's amplicon that not seen in the Razi's field isolate.

Finally based on HRM curve analysis and GCP, five specimens were found as new IBV variants (Table 3).

Table 3: Classification of positive field samples based on visual evaluation of melt curves and GCP (> 95%).

Field specimen's HRM analysis group	Sample Code	Genotype
1	F.01- F.02	793/B
2	F.03- F.04	793/B
3	F.05- F06- F.07	New Variant
4	F.11- F.19	New Variant
5	F.12	793/B
6	F.20	793/B

DISCUSSION

Since the first detection of IBV in Iran (Aghakhan et al., 1994), several studies were done to evaluate the situation of IB outbreaks. Recent studies reported that 793/B is the dominant strain in Iranian poultry flocks (Seyfi Abad Shapouri., 2004; Shoushtari., 2008). In order to establish a successful vaccination schedule, as a most important part of prevention strategies, differentiation of IBV is so critical and currently performs according to S1 gene sequencing. The main problem of S1 gene sequencing as a golden standard method for IBV differentiation is a high propensity of this gene to mutation even in multiple passages and field circulation or be a probable site of recombination (Capua., 1999; Li., 2010) (4). Moreover other limitations such as lack of reliable pair of primers for amplification of S1 gene for all IBV strains and large size of its PCR products, which may lead to false negative results, have been reported for this method (Hewson., 2010). To set a reliable method for differentiating IBV, other part of IBV's genome such as 3' UTR have been suggested and some pairs of primers have been introduced for this fragment (Adzhar., 1996). The IBV 3' UTR hyper-variable region was used for HRM analysis, as it appears less prone to spontaneous mutation than S1 gene and because of its small size of 3'UTR amplicons (Hewson., 2010). Moreover, a strong inter-strain correlation between the S1 gene and the hyper-variable region in the 3'UTR assists in validating the use of the 3'UTR for IBV strain differentiation (Hewson., 2010). It has been reported that IBV vaccine strains can be differentiated from field infections due to occurrence of deletion in 3'UTR sequenc-

es detected by Restriction Fragment Length Polymorphism (RFLP) and HRM analysis (Hewson., 2009; Hewson., 2010; Majdani., 2012; Mardani., 2006).

Following recent outbreaks of IB infection in Iranian poultry farms despite vaccination and due to presence of some restrictions in current diagnostic techniques for IBV identification and differentiation, such as time consuming and the high cost of operation, this study conducted to evaluate ability of HRM analysis in differentiation of current Iranian's IBV strains. Previous studies about application of HRM technique mentioned that this method is much faster than conventional DNA sequencing and suggested 3' UTR HRM curves analysis as a useful method to detect and differentiate IBV strains (Hewson., 2009; Hewson., 2010).

In current study, detection and identification of IBV strain by HRM analysis done for the first time in Iran. HRM analysis in conjunction with GCPs can differentiate current live IB vaccine in Iran from field isolates and a useful curve melt pattern demonstrated for those mentioned references. Moreover, a 18 bp deletion found in the first part of 3'UTR PCR products of 793/B vaccine strains, which not happened in field reference of 793/B strain that led to different melt curve pattern by which these vaccine strains can be differentiated from the same field serotypes, that was experienced previously in a Australian study which reported that a 58 bp deletion found in current Australian vaccine comparing by field isolates (Hewson., 2009; Mardani., 2006).

Finding of 5 specimens by above 95% similarity to

confirmed QX-like strains was indicating presence of new variant in Iranian poultry farm. This result can be a probable cause of vaccination failure in the premises. Before this finding, many poultry veterinarians reported similar clinical and post mortem lesion of QX-like strains in flocks (reports not published) which seen despite of extreme use of Massachusetts vaccines in farms. One study mentioned that due to consistent use of Massachusetts vaccines from 1997 in Sweden, genotyping variation occurred in circulating IB strains, which leaded to vaccination failure (Farsang., 2002) that may refer to the same phenomenon in Iran. Moreover there are many reports of QX-like in the European country which indicates wide spread of this new variant around the world (Beato., 2005; Bochkov., 2006; Worthington., 2008).

This study concluded the molecular presence of QX-like variants in Iranian poultry flock by using HRM curve analysis, as a new reliable, fast and cost benefit molecular technique and it is suggested that in order to complete genotyping of these new strains, other procedure such as viral isolation and S1 sequencing for phylogenic studies would be preferable to confirm statement.

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