Designing a new primer based on the E gene for more accurate molecular detection of avian infectious bronchitis virus

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Abstract

Background:

Infectious bronchitis (IB) is a highly contagious disease of chickens caused by the infectious bronchitis virus (IBV), affecting the respiratory and urogenital systems and leading to significant economic losses due to decreased egg quality and increased susceptibility to secondary infections. Early and reliable molecular diagnosis of IBV is essential for effective disease control. Although conserved genomic regions such as the N gene and untranslated regions (UTRs) are commonly targeted in diagnostic assays, alternative conserved targets may improve detection performance.

Materials and Methods:

In this study, a novel primer targeting a conserved region of the E gene of the IBV genome was designed to amplify a 147-bp fragment. Its performance was compared with a previously reported N-gene-based primer. Equal concentrations of viral RNA from four IBV strains (Ma5, Variant 2, QX, and 793/B) were extracted and subjected to RT-PCR using both E- and N-gene primers. Three RNA concentrations (undiluted, 1:10, and 1:100 dilutions) were evaluated to assess detection sensitivity.

Results:

RT-PCR results showed that the E-gene primer successfully detected QX and Ma5 strains at all three concentrations (undiluted, 1:10, and 1:100). The 793/B strain was detected at undiluted and 1:10 dilutions, while Variant 2 was weakly detected only in the undiluted sample. In contrast, the N-gene primer weakly detected only the 793/B strain at undiluted and 1:10 dilutions and failed to detect the other strains at any concentration.

Conclusion:

The newly designed E-gene primer demonstrated superior detection performance compared to the previously used N-gene primer. Targeting conserved regions of the IBV genome, such as the E gene, may enhance the sensitivity and reliability of molecular diagnostic assays. Further optimization and validation studies are recommended to improve IBV detection strategies.

Keywords: Infectious Bronchitis Virus, Iran, RT-PCR, Primer

1. Introduction

Infectious bronchitis, caused by Infectious Bronchitis Virus (IBV), is a highly contagious condition in chickens that mainly affects the respiratory tract and may also involve the urogenital systems [1, 2]. The disease leads to respiratory symptoms, reduced growth performance and feed efficiency in broilers, and declines egg production and shell quality in layers [1]. Infectious bronchitis can cause significant economic losses, as it has been identified as the second detrimental disease in the poultry industry after highly pathogenic avian influenza [3, 4]. Infectious bronchitis virus belongs to the genus *Gammacoronavirus* within family *Coronaviridae* and order *Nidovirale*. Its genome consists of, positive-sense, single-stranded RNA of approximately 27–28 kb. The genomic organization follows the order as *5´-UTR-1a/1b-S-3a-3b-E-M-4b-4c-5a-5b-N-6b- 3'UTR* and encodes for different proteins [5, 6]. Non-structural proteins (nsp) are encoded by open reading frames 1a and 1b and are initially translated as two polyproteins through a -1 ribosomal frameshift. After translation these polyproteins cleaved to different nsps through autoproteolytic activity [7]. Following this part, genome also encodes for different important proteins such as Spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. In addition, virus genome consists two accessory gens, ORF3 and ORF5, encoding proteins 3a, 3b, 5a and 5b [5]. The E glycoprotein (small envelope protein), encoded by the 3C gene, is a minor viral envelope protein (8–12 kDa) that, although not essential for replication, is crucial for viral assembly, budding, and infectivity in conjunction with the M protein [8, 9]. The N protein, a structural protein of 45–50 kDa, binds the viral RNA to form the helical ribonucleoprotein (RNP) and plays key roles in virus replication, assembly, and host immune response [8, 10].

Different IBV genotypes are reported in Iran like Massachusetts (Mass), 793/B, QX, Variant 2 (IS-1494 like), IR1 and IR2, with different prevalence rate [11]. The S glycoprotein, responsible for fusion of viral envelope and host cell membrane, consists of two part, S1 and S2 which are proteolytically cleaved into separate subunits. S1 subunit which carries the virus-neutralizing and serotype-specific determinants, reveals high sequence variability than S2 subunit, and because of this, characterization and detection of IBV is mainly relied on analysis of variable S1 gene or the expressed S1 protein [12]. Furthermore, molecular detection sequencing and analysis of Iranian isolates of IBVs based on S, M, N and 3' UTR were done in earlier molecular researches [13-15].

Detection of IBV can be done through different approaches like virus isolation, tissue culture, immunohistochemistry, in situ hybridization, direct or indirect fluorescent antibody and enzyme-linked immunosorbent assay (ELISA), and also molecular assays like RT-PCR [11, 16]. For detection of IBV, molecular assays are commonly used due to their specificity, sensitivity and rapid results. These assays utilize reverse transcriptase-polymerase chain reaction (RT-PCR) to find viral RNA in either clinical samples or from viruses cultivated in laboratory [16]. In present study we developed a novel pair of primers designed based on E gene for more accurate detection of IBV strains via RT-PCR molecular assay.

2.Material and Methods

2.1 Virus isolation and RNA extraction

In this study, four strains of Infectious Bronchitis Virus (Ma5, Variant2, QX, and 793/B) were selected. The QX and Variant 2 strains were previously isolated by Ghalyanchi Langeroudi et al [17] while commercial vaccines were used as sources for the Ma5 and 793/B strains. Total RNA from all samples was extracted using SinaPure™ RNA extraction Kit (SinaClon, Iran) following the manufacturer's instructions. The extracted RNA was eluted in 50 µl of elution buffer and quantified using NanoDrop One/One^{c} (Thermofisher Scientific) (Table 1).

Table 1. The concentration of four strains of IBV extracted RNA with nanodrop

IBV Strain	Concentration	Unit	A260	A280	A260/280	A260/230
Ma5	7.707	µg/ml	0.193	0.146	1.315	0.018
793/B	9.164	µg/ml	0.229	0.151	1.518	0.013
Variant2	5.558	µg/ml	0.139	0.108	1.282	0.018
QX	8.832	µg/ml	0.221	0.149	1.484	0.026

2.2 Preparation of Primers

Novel primers in this study were designed to target a partial sequence of E protein encoding gene of infectious bronchitis virus. For this purpose, a dataset of previously submitted IBV sequences from GeneBank was compiled. All sequences in the dataset were aligned using MEGA7 software and sequences with poor quality (containing ambiguous nucleotides) were removed. The curated dataset was then screened to identify conserved regions in both directions (5' to 3' and 3' to 5')

suitable for primer design. The designed primers were synthesized by SinaClon (Tehran, Iran). The novel designed primers named "IBV EZGF" and "IBV EZGR", were compared with previously described primers N103F and N102R (Table 2) [18]. The positions and coverage of the primers across the four selected IBV strains are presented in Figure 1.

Table 2. The sequences of primers amplifying E and N genes used in this study

Primer Name	Primer Sequence (5′⟶ 3′)	Size (bp)	Target Gene	Reference
IBV EZGF	GTTTATTTTGGTATACATGG	145	Gene E	This study
IBV EZGR	TTATTATTCCAACCGTTC			
N103F	CCTGATGGTAATTTCCGTTGGG	357	Gene N	[18]
N102R	ACGCCCATCCTTAATACCTTCCTC			

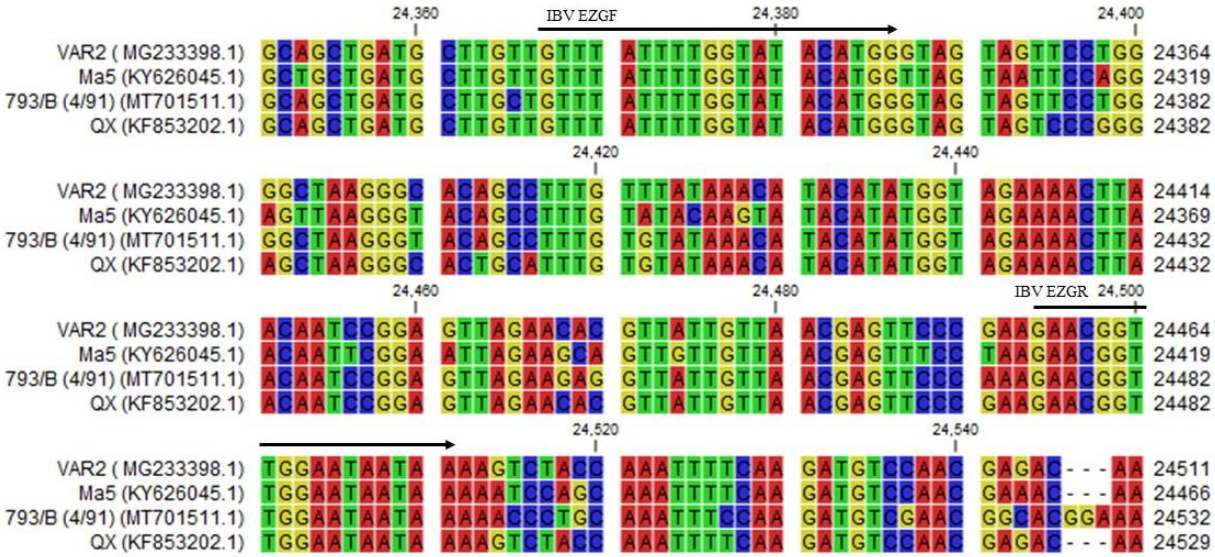

Figure 1 – partial sequence alignment of 4 selected strains in this study included for designing primer targeting E protein encoding gene. Arrows above alignment shows the position of binding primers. The name of primers is shown above arrows.

2.3 cDNA synthesis and RT-PCR

Extracted RNA was immediately used for cDNA synthesis, and the remaining aliquots were stored at −70 °C for subsequent experiments. Based on NanoDrop quantification, equal amounts of RNA

were used for all strains during cDNA synthesis. Reverse transcription was performed using the Viva cDNA Synthesis Kit (VIVANTIS TECHNOLOGIES, Malaysia) according to the manufacturer's protocol. The synthesized cDNA was subsequently stored at −20 °C for further assays.

For PCR assays, three different dilutions (1:1, 1:10, and 1:100) of each strain's cDNA were prepared. The PCR reaction mixture (25 µL) contained 3 µL of cDNA, 1 µL each of forward (IBV EZGF) and reverse (IBV EZGR) primers, 12 µL of VIVANTIS Master Mix (VIVANTIS TECHNOLOGIES, Malaysia), and 8 µL of nuclease-free water. The E gene was amplified using the following cycling conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 48 °C for 10 s, and 68 °C for 15 s, with a final extension at 72 °C for 7 min. Amplification of the N gene by RT-PCR was performed under these conditions: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s. A final extension was conducted at 72 °C for 7 min. RT-PCR products then finally were electrophorized in 1.5 % agarose gel in bromide-containing Tris-acetate-EDTA buffer for 45 min at 100 V, followed by visualization under ultraviolet light.

3.Results

According to PCR results, the E gene targeting primes (IBV EZG F/R) produced sharp and distinct bands for the QX, 793/B and Ma5 strains, while Variant 2 was only weakly detected at the 1:1 concentration. In contrast, the N gene targeting primers yielded only a faint band for the 793/B strain at the same concentration. At the 1:10 dilution, the E gene primers consistently detected QX, 793/B, and Ma5 strains, but no band was observed for Variant 2. At this dilution, the N gene primers produced only a faint band for the 793/B strain. At the 1:100 dilution, the E gene primers detected only QX and Ma5, whereas the N gene primers failed to detect any strain. These results are summarized in Table 3, and representative agarose gel images are presented in Figure 2.

145 **Table 3.** Comparative results of RT-PCR assay using two pairs of primers targeting E and N gene
146 in 3 different concentrations of cDNA for four strains of IBV.

Genotype	Concentration	E gene primer	N gene primer
QX	1	+	-
	1/10	+	-
	1/100	+	-
Variant 2	1	Weak +	-
	1/10	-	-
	1/100	-	-
Ma5	1	+	-
	1/10	+	-
	1/100	+	-
793/B	1	+	Weak +
	1/10	+	Weak +
	1/100	-	-

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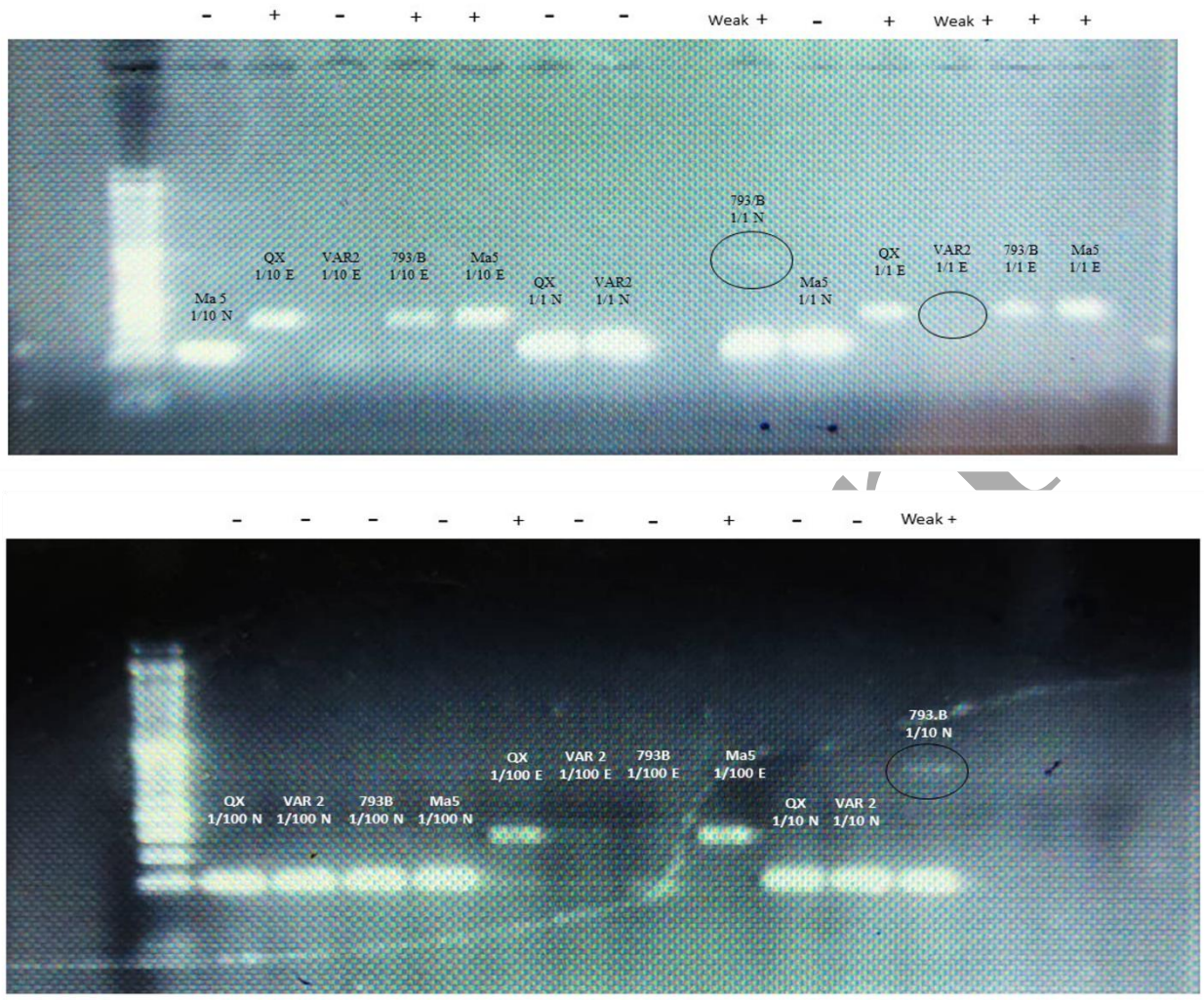

Figure 2 – Agarose gel electrophorises image. The names of the strains and primers used are indicated on the image. "E" represents amplification with IBV EZG F/R primers, and "N" corresponds to amplification with N102/103 primers. Weak bands are highlighted with black circles. The three cDNA concentrations used in each lane are also labeled on the image.

4.Discussion

IBV is a highly contagious poultry pathogen, and outbreaks continue worldwide despite vaccination, leading to economic losses. In Iran, the circulation of diverse strains complicates control due to genetic and antigenic variability [11, 19, 20]. Thus, reliable molecular assays are essential for accurate detection.

Among the available diagnostic tools for IBV, molecular detection is one of the most effective. In this study, we applied this approach to evaluate the performance of E and N gene primers. Until

now, several primer pairs have been proposed for the detection and classification of IBV. The choice of primers is closely related to the specific objective of detection. For instance, the S1 subunit of the spike (S) glycoprotein has traditionally been used to determine IBV serotypes [19]. However, other regions of the genome have also been targeted for virus classification. For example, in 2009 Hewson et al, developed an RT-PCR high-resolution melt curve analysis based on the 3′ UTR of IBV, which enabled rapid detection and classification of the virus in commercial poultry [20]. In another study in 2002, Farsang et al. developed a nested RT-PCR assay using two primer pairs targeting the nucleocapsid gene of IBV, along with an additional pair amplifying an 840 bp region of the S1 gene. Their results showed that N gene primers successfully detected all clinical and reference strains, whereas the S1 gene primers failed to detect two samples. This failure was attributed either to low RNA concentration in the samples or to mismatches between the primers and the viral S1 gene [21]. Domanska-Blicharz et al, in 2014, used a real-time RT-PCR assay targeting the 5′ UTR and identified 31 positives. When re-tested with primers for the 3′ UTR, RdRp, and N genes, only 10 were confirmed. This discrepancy was attributed to the higher sensitivity of real-time RT-PCR or sequence variability affecting primer binding [22]. In another study, Sun et al also developed a one-step RT-PCR assay targeting the N gene for IBV detection. This method successfully detected multiple serotypes, including Massachusetts, 4/91, LX4, CK/CH/LSC/991, CK/CH/LDL/971, and Connecticut. However, they noted that slight primer modifications may be required to improve detection across additional serotypes [23].

In Iran, different studies also designed primers for molecular detection of IBV and its genotypes. In a study, Ghalyanchilangeroudi et al designed and suggested genotype specific primers for detecting Variant 2, QX, D274 AND 793/B. However, they used a nested RT-PCR assay using primers targeting S gene which previously used by Worthington et al for sequencing and phylogenetic study [11]. In another study in Iran, Madhi et al, developed a RT-PCR assay by designing primers, targeting a 129bp fragment of 5' UTR region of IBV genome and by this method they suggested a time saving, sensitive and reliable assay for detecting IBV [24].

The envelope (E) gene has been also utilized as a target for the molecular detection of human coronaviruses. In a study in 2023, Yu et al demonstrated a designed RT-qPCR assays targeting the E gene which yield high specificity, with no cross-reactivity to other human coronaviruses or common respiratory viruses [25]. The E gene, due to its short length and high conservation across

SARS-CoV and SARS-CoV-2, can serve as an effective target for primary screening in RT-qPCR assays [26].

These results emphasize the importance of designing primers that target conserved regions of the viral genome to increase detection across diverse strains. In the present study, we designed primers targeting a conserved region of the E gene and compared them with conventional N gene primers. RT-PCR results showed that the E gene primers efficiently detected QX and Ma5 strains at all tested concentrations, detected 793/B at 1 and 1:10 dilutions, and weakly detected Variant 2 only in undiluted samples, likely due to low RNA concentration. In contrast, N gene primers only weakly detected 793/B and failed to detect the other strains.

5.Conclusion:

Taken together, the findings of this study indicate that the newly designed primer targeting a conserved region of the IBV E gene represents an effective approach for detecting diverse IBV strains and demonstrates superior performance compared to the conventional N-gene primer evaluated in this study. These results highlight the importance of targeting conserved genomic regions and support further development and optimization of such primers to enhance the sensitivity and reliability of molecular diagnostic assays for IBV.

6. Acknowledgement

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7. Grant support or Funding

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8.Conflict of interests

The authors declare no conflict of interests.

9. Authors Contributions

Primer design and analysis: A.G.L, Z.Z.K

Manuscript writing: Z.Z.K, M.R.G, S.S

Study supervision: A.G.L

Final review and editing: A.G.L

10. Data availability

The data that supporting the findings of this study are available upon request from the corresponding author.

11. Ethics committee Approval

Our study did not involve any invasive procedures on animals therefore, an ethics committee approval is unnecessary.

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طراحی یک پرایمر جدید بر اساس ژن E برای تشخیص مولکولی دقیق‌تر ویروس برونشیت عفونی پرندگان

چکیده

زمینه مطالعه:

برونشیت عفونی (IB) یک بیماری بسیار واگیردار در طیور است که توسط ویروس برونشیت عفونی (IBV) ایجاد می‌شود و دستگاه‌های تنفسی و ادراری-تناسلی را درگیر می‌کند. این بیماری با کاهش کیفیت تخم‌مرغ و افزایش حساسیت به عفونت‌های ثانویه، موجب خسارات اقتصادی قابل‌توجهی می‌شود. تشخیص مولکولی سریع و قابل‌اعتماد IBV برای کنترل مؤثر بیماری ضروری است. اگرچه در آزمون‌های تشخیصی معمولاً نواحی ژنومی محافظت‌شده‌ای مانند ژن N و نواحی غیرترجمه‌شونده (UTRs) هدف قرار می‌گیرند، استفاده از سایر نواحی محافظت‌شده ممکن است عملکرد تشخیصی را بهبود بخشد.

موارد و روش کار:

در این مطالعه، یک جفت پرایمر جدید با هدف ناحیه‌ای محافظت‌شده از ژن E ژنوم IBV طراحی شد که قطعه‌ای به طول ۱۴۷ جفت‌باز را تکثیر می‌کند. عملکرد این پرایمر با یک پرایمر مبتنی بر ژن N که پیش‌تر گزارش شده بود مقایسه گردید. غلظت‌های مساوی RNA ویروسی از چهار سویه IBV شامل Ma5، Variant 2، QX و B/793 استخراج و با استفاده از هر دو پرایمر ژن E و ژن N تحت آزمون RT-PCR قرار گرفتند. به‌منظور ارزیابی حساسیت تشخیص، سه غلظت RNA شامل نمونه رقیق نشده، رقت ۱:۱۰ و ۱:۱۰۰ مورد بررسی قرار گرفت.

نتایج:

نتایج RT-PCR نشان داد که پرایمر ژن E سویه‌های QX و Ma5 را در هر سه غلظت (بدون رقت، ۱:۱۰ و ۱:۱۰۰) با موفقیت شناسایی کرد. سویه B/793 در نمونه‌ی رقیق نشده و غلظت ۱:۱۰ شناسایی شد، در حالی‌که سویه Variant 2 تنها در نمونه رقیق نشده به صورت ضعیف تشخیص داده شد. در مقابل، پرایمر ژن N تنها توانست سویه B/793 را در نمونه‌ی رقیق نشده و غلظت ۱:۱۰ به‌صورت ضعیف شناسایی کند و موفق به شناسایی سایر سویه‌ها در هیچ‌یک از غلظت‌ها نشد.

نتیجه گیری:

پرایمر جدید طراحی‌شده بر پایه ژن E در مقایسه با پرایمر مبتنی بر ژن N که پیش‌تر مورد استفاده قرار می‌گرفت، عملکرد تشخیصی بهتری نشان داد. هدف‌گیری نواحی محافظت‌شده ژنوم IBV، مانند ژن E، می‌تواند حساسیت و قابلیت اطمینان آزمون‌های تشخیص مولکولی را افزایش دهد. انجام مطالعات بیشتر به‌منظور بهینه‌سازی و اعتبارسنجی این پرایمرها برای بهبود راهبردهای تشخیص IBV توصیه می‌شود.

کلمات کلیدی: : ویروس برونشیت عفونی، ایران، RT-PCR، پرایمر