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## Original Article

# Expression Study of Subgenomic mRNAs of Avian Infectious Bronchitis, Variant 2, (GI-23), using RNA-Seq Analysis

**Ziafati Kafi Z[1], Molouki A[2], Hosseini H[3], Sadri N[1], Sarmadi S[1], Fallah Mehrabadi MH[2], Tolooe A[1], Ghalyanchilangeroudi A[1*]**

1. Department of Microbiology and Immunology, University of Tehran, Faculty of Veterinary Medicine, Tehran, Iran.
2. Department of Avian Diseases Research and Diagnostics, Razi Vaccine and Serum Research, Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.
3. Department of Clinical Sciences, Faculty of Veterinary Medicine, Karaj Branch, Islamic Azad University, Alborz, Iran.

## Abstract

**Background and Aims:** Infectious bronchitis virus (IBV), a member of the genus Gamma coronavirus under the Coronaviridae family, is one of the leading causes of persistent economic and financial burdens poultry industry of Iran. This family of viruses possesses a ~27 kb positive-sense RNA genome that produces a nested set of subgenomic mRNAs during replication.

**Materials and Methods:** This study investigated tissue tropism of virulent IBV variant 2, GI-23, by comparing viral gene expression differences between kidney and trachea tissue samples of 20 intranasal infected SPF chickens. At 2 and 6 days post-infection, extracted genomic RNA was subjected to RNAseq, and high-throughput analyses studied the obtained data.

**Results:** Among the genomic UTRs of IBV, the highest expression was obtained for the 3'UTR (7.15E+05) in the renal samples. Nucleoprotein (N) had the 2nd highest gene expression in both tissues (4.52E+05 in the trachea and 4.13E+05 in the kidney). The lowest expression (2.34E+03 in the trachea and 2.69E+02 in the kidney) belonged to the polymerase genes. 5'UTR expression was not detected in any of the tissue samples.Furthermore, six non-structural genes (NSP4, 7, 8, 9, 11, and 15) had no detectable expression in the kidney samples. NSP11 was shared in both tissues in this case, while NSP16 had the highest expression at 1.62E+05 in tracheal samples and NSP13 at 2.88E+5 in renal samples.

**Conclusion:** We concluded that except for the 3' UTR, a slightly higher expression was observed in tracheal samples.

**Keywords:** Avian Infectious Bronchitis, RNA-Seq Analysis, Variant 2, IBV genome

## Introduction

Coronaviruses (CoVs) can cause severe diseases in several avian and mammalian species, such as humans. Genetically, CoVs belong to the order Nidovirales, the family of Coronaviridae, and the subfamily of Orthocoronavirinae. The Orthocoronavirinae subfamily itself is further divided into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and the recently identified Deltacoronavirus (1).

Currently, there are two known species under the Gammacoronavirus genus (namely avian coronavirus (ACoV) and beluga whale coronavirus SW1 (Whale-CoV SW1), which is relatively less than those under the other genera (2). ACoVs infect several avian hosts and include some important poultry pathogens such as infectious bronchitis virus (IBV) and turkey coronavirus (TCoV)(3). The number of serotypes of ACoV has exceeded 60, which is an outcome of mutation in its large genome.

Selecting a good vaccine against IBV is critical due to the poor cross-protection among the different IBV serotypes (4). Based on the sequence of the hypervariable region (HVRs) of the S1 gene, seven phylogenetically distinct

[*]**Corresponding author:**
Arash Ghalyanchilangeroudi,
Email: ghalyana@ut.ac.ir.

groups IBV (namely Mass, 793/B-like, IS/1494-like, IS/720-like, QX-like, IR-1, and IR-2) have been identified in Iran (5). Despite using various vaccines, such as mass & 793/B in Iranian poultry farms, IB outbreaks and high mortality rates are still big issues.

One of the novel genotypes of IBV, circulating predominantly in the Middle Eastern countries, is the variant 2 (IS-1494/06-like GI-23) genotype (6). The presence of the variant 2 viruses in Iranian commercial flocks has been previously reported (5). Variant 2 IBV is nephropathogenic, but it also affects the respiratory system (7).

The IBV is an enveloped virion that contains a positive-sense, non-segmented, single-stranded RNA of approximately 27.6 kb in length.

The IBV viral genome consists of a capped 5' untranslated region (5'UTR), a leader sequence, two open reading frames (ORF) of 1a/b encoding non-structural proteins NSP, four structural proteins, namely the spike (S), envelop (E), membrane (M) and nucleoprotein (N), accessory elements like the ORF of 3a, 3b, 5a, 5b, a 3′ untranslated region (3'UTR) and finally a poly-A tail. Each component of the IBV genome has a different role.

The two overlapping ORFs of 1a and 1b, constituting approximately two-thirds of the genome, are translated as the large polyprotein 1ab associated with RNA replication and transcription. The spike protein of IBV is cleaved into S1 and S2 glycoproteins; however, the S1 subunit is the major target of neutralizing antibodies and contains serotype-specific antigenic determinants.

The C-terminal portion of the S2 protein intercalates in the virus envelope and helps the S1 protein anchor in the membrane. The E and M proteins are membrane-associated proteins that are needed to form virus-like particles and virus budding. Furthermore, an association between the N protein and RNA genome forms the ribonucleoprotein (4).

Despite routine vaccination, the prevalence of IBV variant 2 is still high in Iran. Therefore, in this study, variant 2 in two chicken tissues, namely kidney, and trachea, was investigated using an RNAseq.

# Methods

**Ethics Statement:** All of the work was carried at the University of Tehran according to the Ministry of Health and the Medical Education of Iran for ethical conduct in the care and use of animals in research and authorized by the Institutional Animal Care and Use Committee (IACUC).

**Experimental Design:** Twenty-one-day-old specific pathogen-free (SPF) White Leghorn chickens were provided by Razi Vaccine and Serum Research Institute of Iran. The chickens were kept in positive-pressure isolators for 22 days and then inoculated with 0.1 ml of $10^{4.5}$ EID50 of IBV Variant 2 by oculonasal inoculation at 14 days old. The trachea tissue samples (10 chickens) were collected at 2 days post-inoculation (dpi), and the kidney tissue samples (10 chickens) were collected at 6 dpi.

**RNA extraction:** RNA was extracted from sampled tissues (trachea and kidney) separately using Trizol (Roche, Germany, Cat No: 12033674001) per the manufacturer's protocol. DNase I (Ambion, Austin, TX) digestion was performed following RNA isolation, and then the RNA concentration and purity were determined by NanoDrop ND-1000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA). RNA samples of each tissue were pooled together and stored at -80 °C for later use. The RNA quality was further assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

**RNA Sequencing:** Based on the instructions provided by the Beijing Genome Institute (BGI; Shenzhen, China), cDNAs for use in RNAseq were synthesized. First-strand cDNA was synthesized using short fragments as templates and random hexamer primers.

Second-strand cDNA was synthesized using dNTPs, RNaseH, DNA polymerase I, and its buffer according to BGI instruction.

Short double-stranded cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, Venlo, Netherlands), resolved with EB buffer for the ends repair and poly-(A) addition, and then ligated to sequencing adapters.

**Table 1.** Expression value (RPKM) of different subgenomic mRNAs of IBV variant 2 in the tracheal and renal samples of the current study's SPF chickens.

| Gene | Trachea | Kidney |
|---|---|---|
| 5' UTR | 0.00E+00 | 0.00E+00 |
| Polymerase | 2.34E+03 | 2.69E+02 |
| Spike | 2.03E+04 | 5.51E+03 |
| 3a | 3.04E+03 | 0.00E+00 |
| 3b | 1.25E+04 | 6.41E+03 |
| 3c | 4.51E+04 | 2.11E+04 |
| Membrane | 9.05E+04 | 3.21E+04 |
| 5a | 8.14E+04 | 5.70E+04 |
| 5b | 2.06E+05 | 1.43E+05 |
| Nucleoprotein | 4.52E+05 | 4.13E+05 |
| 3' UTR | 2.96E+05 | 7.15E+05 |

**Table 2.** Expression value (RPKM) of different NSPs of IBV Variant 2 in the trachea and kidney samples of SPF chickens of the current study.

| Non-Structural | Trachea | Kidney |
|---|---|---|
| NSP2 | 1.56E+04 | 1.90E+04 |
| NSP3 | 1.94E+04 | 6.09E+03 |
| NSP4 | 3.62E+04 | 0.00E+00 |
| NSP5 | 3.09E+04 | 4.38E+04 |
| NSP6 | 3.24E+04 | 1.54E+05 |
| NSP7 | 5.37E+04 | 0.00E+00 |
| NSP8 | 6.62E+04 | 0.00E+00 |
| NSP9 | 5.56E+04 | 0.00E+00 |
| NSP10 | 2.81E+04 | 5.46E+04 |
| NSP11 | 0.00E+00 | 0.00E+00 |
| NSP12 | 2.62E+04 | 1.36E+04 |
| NSP13 | 9.27E+04 | 2.88E+05 |
| NSP14 | 8.13E+04 | 9.00E+04 |
| NSP15 | 1.13E+05 | 0.00E+00 |
| NSP16 | 1.62E+05 | 2.12E+05 |

Suitable enriched fragments were sequenced using a HiSeq 2000 instrument (Illumina, San Diego, CA, USA).

**Bioinformatics Analysis:** Data were recorded in the FASTQ format and then analyzed in CLC Genomics Workbench (version 6.0.1) (CLC Bio, Aarhus, Denmark, Trail). First, the sequence reads were mapped to the IBV variant 2 (MG233398.1) reference genome using the RNAseq mapping algorithm. The highest mismatches count for the mapping was set at 1. Next, the reads per kilobase of the transcript, per million mapped reads (RPKM), were calculated to estimate the gene expression levels described previously (8), and then the expression value was analyzed for each gene.

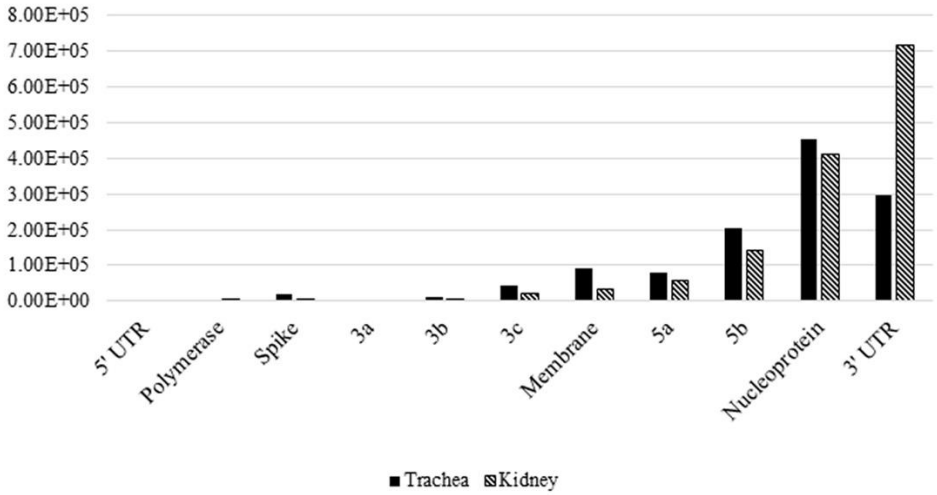



**Fig. 1.** Comparison of an expression value (RPKM) of different subgenomic mRNAs of IBV Variant 2 in SPF chickens' trachea and kidneys.

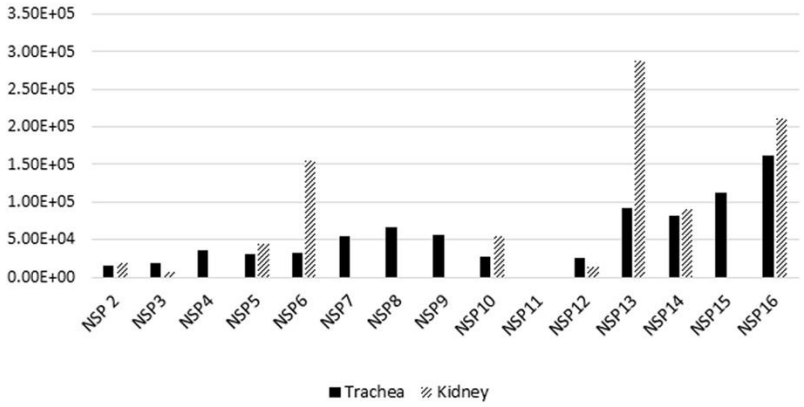



**Fig. 2.** Comparison of an expression value (RPKM) of different non-structural genes of IBV variant 2 in SPF chickens' trachea and kidneys.

## Results

All the data obtained by bioinformatics analyses are shown in Tables 1 and 2, and the corresponding figures are presented in Fig. 1 and 2. The highest gene expressions were detected in the trachea for the N Protein (4.52E+05) and 3'UTR subgenomic mRNA (2.96E+05). In the kidney, these amounts were 4.13E+05 and 7.15E+05, respectively. The lowest expression value was detected for the polymerase genes in both tissues (see Table 1). Additionally, no detectable expressions for the 5' UTR were obtained in either of the tissues. Moreover, in the kidney, the 3a had no detectable expression (Table 1). Among the NSPs, NSP16 and NSP13 had the highest expression values (1.54E+05 and 2.88E+0.5, respectively) in the renal tissues (Table 2, Fig. 2). NSP16, however, had the highest value in the tracheal samples (1.62E+05). However, many other NSPs showed no detectable expression, especially in the kidney. NSP11 had no expression in either of the tissues, surprisingly.

## Discussion

Coronaviruses (CoVs) have been detected in a large group of animals and species. Usually, these viruses cause respiratory, enteric, and nephrological diseases with variable severity. Furthermore, recent IBV isolates, found in different parts of the world (9), have great antigenic diversity, different tissue tropism, and pathogenicity.

In this study, the expression of subgenomic mRNAs in two IBV-infected chicken tissues was investigated. The N protein showed the highest gene expression in both the tracheal and kidney tissues. This protein is involved in different aspects of virus replication (10). Its length varies from 377 to 455 amino acids in different ACoVs. However, it also has other characteristics, such as being highly basic and having a high serine content (7 to 11%), which can be potential phosphorylation targets. Conserved sequences of the N protein within this Gammacoronavirus genus (?) are low (11). This phosphoprotein can bind to viral RNA with high affinity and is widely expressed during infections (12). It is also a frequent target for diagnostic purposes, such as PCR (13). Also, it can be considered as a target protein for vaccine design. CoVs generally down-regulate host gene expression and increase host mRNA degradation (14).

The N protein interferes with the activation of 2′,5′-oligoadenylate synthetase/RNase L (2′–5′ OAS) that causes Type I IFN induction. Besides, the global translational shutdown inhibits the generation of different proinflammatory cytokines and chemokines (15). The N gene′s high expression in our IBV Variant 2 strain may indicate that it facilitates the virus escape from the host immune system, resulting in more replication rate in the corresponding tissues. However, this statement requires further investigation.

In this study, the lowest expression belonged to the polymerase genes in both the kidney and trachea tissues. mRNA 1 contains ORF1a and ORF1b that encode two large polyproteins through a ribosomal frameshift mechanism (16). As a multifunctional protein, the replicase polyprotein exhibits activities needed for the transcription of negative-stranded RNA, progeny virion RNA, leader RNA, subgenomic mRNAs, and expression of proteinases in charge cleaving the polyprotein into functional products (17). After that, two virus-encoded proteases, namely the papain-like protease encoded in NSP3 and the 3C-like (main) protease encoded in NSP5, auto-proteolytically process the cleaved polyproteins into 16 NSPs (18). Unlike the viral structural proteins, the functions of NSPs are associated with the modulation of the host environment to make viral RNA synthesis and viral replication feasible. NSP16 had the highest expression in tracheal tissues and the second highest in kidney samples of this study. NSP16, a 2′O-methyltransferase, recognizes and binds to cap-0 RNA to generate methylated cap-1. NSP16 is one of the most conserved proteins of coronaviruses (reference). On the other hand, NSP13 had the highest expression in the kidney samples.

The Nsp13 helicase protein is associated with enzymatic activities such as RNA and DNA duplex-unwinding activities, RNA 5′-triphosphatase activity, and NTPase and dNTPase activities that can have a role to play in the formation of the 5′-cap structure of viral RNAs (19), the methyltransferase activities of nsp14 and nsp16 also nsp16 formation of RNA cap (4, 20), the exoribonuclease activity of nsp14 and the endoribonuclease activity of nsp15 (20).

In this study, 3’UTR had the highest expression in both of the tissues. The 5’ and 3’ UTRs have structural motifs that play a role in the interactions between the UTRs and viral encoded replicase proteins and even the host proteins (reference). Thus, the UTRs have a major role to play in viral RNA transcription and replication. In addition, researchers have suggested that regions in the 3′ UTR have cis-acting regulatory elements that contribute to coronavirus RNA synthesis (reference). These regions adjoin the poly (A) stretch and may form many diverse stem-loop structures.

## Conclusion

This study was conducted on IBV variant 2, one of the most predominant and virulent strains of IBV in Iran (11). According to the results of this study, the transcription level of IBV genes may help the virus replicate better in a particular tissue.

However, further assessments are required to thoroughly understand how IBV counteracts birds’ immune systems to replicate and cause infection.

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## Conflict of interest

No conflict of interest is declared.

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