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1 Analysis of synonymous codon usage in spike protein gene of infectious bronchitis virus

2 Aditi Makhija¹, Sachin Kumar^{1*}

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4 ¹Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati,
5 Guwahati, Assam 781039, India.

6 * Corresponding author: Mailing address: Department of Biosciences and Bioengineering,
7 Indian Institute of Technology Guwahati, Guwahati, Assam 781039, India.

8 Phone: (91) 3612582229; Fax: (91) 3612582249

9 E-mail: sachinku@iitg.ernet.in

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Abstract

Infectious bronchitis virus (IBV) is responsible for causing respiratory, renal and urogenital disease in poultry. IBV infection in poultry lead to high mortality rates in affected flocks and severe economic losses due to drop in egg production and reduce gain in live weight of the broiler birds. IBV encoded spike protein (S) is the major host protective immunogen. Although the functions of the S protein have been well studied, the factors shaping synonymous codon usage bias and nucleotide composition in the S gene have not been reported yet. In the present study, we have analyzed the relative synonymous codon usage and effective number of codons (ENC) using the 53 IBV S genes. The major trend in codon usage variation was studied using correspondence analysis. The plot of ENC values and GC3s as well as the correlation between base composition and codon usage bias suggests that mutational pressure rather than natural selection is the main factor that determines the codon usage bias in S gene. Interestingly, no association of aromaticity, degree of hydrophobicity and aliphatic index has been observed with the codon usage variation in IBV S genes. The study represents a comprehensive analysis of IBV S gene codon usage patterns and provides a basic understanding of the codon usage bias.

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Keywords

Mutational pressure; aromaticity; aliphaticity; IBV; evolution.

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42 **Notes:**

43 Infectious bronchitis (IB) is an acute and highly contagious respiratory disease of
44 poultry that is prevalent throughout the world. Infection of IB can lead to substantial
45 economic losses to the poultry industry worldwide. The causative agent, avian infectious
46 bronchitis virus (IBV) is a group member of the genus *Coronavirus* under family
47 *Coronaviridae*. It is commonly found in the respiratory and non-respiratory tissues such as the
48 epithelial lining of the gut, kidney and oviduct of the domestic fowl (Jackwood 2012). The
49 coronaviruses are enveloped and contain an unsegmented, 5'-capped and 3'-polyadenylated,
50 single-stranded, positive-sense RNA genome of 28–32 kb (Bourisnell et al. 1987). It replicates
51 in the host cell cytoplasm. The spike glycoprotein (S), a small membrane protein (E) and an
52 integral membrane protein (M) are the three major membrane proteins in coronavirus. The S
53 protein is a type I glycoprotein composed of three homopolymers that is responsible for
54 binding to the target cell receptor and fusion of the viral and cellular membranes (Casais et al.
55 2003). The IBV S glycoprotein (1,162 amino acids) is cleaved into two subunits, S1 and S2
56 of 535 and 627 amino acids, respectively.

57 IBV is currently controlled by the use of both live attenuated and inactivated vaccines.
58 The IBV S1 subunit can induce neutralising antibodies that are responsible for protecting
59 birds against subsequent IBV infection and associated IB disease. However, many different
60 IBV serotypes have been reported because of the variations in amino acid sequence of the S1
61 subunit leading to failure of vaccines due to lack of cross-protection (Abro et al. 2012).
62 Moreover, the first and third quarters of the S1 protein is sufficient to induce an effective
63 immune response against IBV infection in birds (Kant et al. 1992). There is overwhelming
64 information suggesting that only a few amino acid differences amongst S proteins are
65 sufficient to have a detrimental impact on cross-protection (Martin et al. 2014; Rimondi et al.
66 2009; Sun et al. 2007). In addition, genetically engineered IBV vaccines with heterologous

67 spike protein genes have shown promising results against its infection in poultry (Cavanagh
68 2007).

69 Viruses such as influenza virus, Newcastle disease virus, infectious bursal disease
70 virus, human cytomegalovirus and porcine reproductive and respiratory syndrome, classical
71 swine fever virus have been extensively studied for synonymous codon usage for their
72 various genes (Deka and Chakraborty 2014; Ding et al. 2014; Hu et al. 2014; Kumar et al.
73 2015; Kumar and Kumar 2014; Tao et al. 2009; Wang et al. 2011). Although the functions of
74 the S protein in IBV have been well studied, but extensive synonymous codon usage study
75 has not been reported. The present study is conducted in order to better understand the
76 characteristics of the IBV S gene and to reveal more information regarding the role of
77 aromatic, aliphatic and hydrophobic amino acids in its codon bias.

78 Codons in genes do not occur at equal frequencies, but follow a synonymous codon
79 usage pattern. In both the prokaryotic and eukaryotic genes, the synonymous codon usage is
80 non-random (Babbitt et al. 2014; Li 1997). It has been postulated that genes in any genome
81 follow the coding pattern in accordance with the synonymous codon and a similar trend of
82 codon usage is seen within an organism and in related species (Butt et al. 2014; Li 1997).
83 Studies on synonymous codon usage depict that they vary not only in different genomes, but
84 also in different genes of the same genome (Grantham et al. 1980). The highly expressed
85 genes have a strong preference for a subset of codons in comparison to the ones that are less
86 expressed which have a uniform set of codon usage (Epstein et al. 2000; Gu et al. 2004). The
87 alternative codons also result in the same protein sequences, but may differ in structure and
88 functions (Li et al. 2011). Factors contributing to codon usage bias include gene expression
89 level, G+C% composition, GC skew, amino acid conservation, protein hydropathy,
90 transcriptional selection, RNA stability, optimal growth temperature and hyper saline
91 adaptation (Ermolaeva 2001; Kober and Pogson 2013; Lynn et al. 2002; Paul et al. 2008).

Fifty three complete sequences of S protein genes of IBV isolates were obtained from GenBank (Table 1). The codon usage pattern analysis was performed in the coding sequences of the S protein gene by inserting a stop codon at the end of every sequence of the gene. The patterns of codon usage were analyzed for 53 S protein gene sequences of IBV. The calculation of relative synonymous codon usage (RSCU) index enables the characterization of synonymous codon usage and is expressed as the ratio of the observed usage of codons to the expected value (Sharp PM 1993; Sharp and Li 1986). The RSCU values of each codon in each gene were used to measure the synonymous codon usage using Codon W 1.4.2 software. The RSCU value of 1 indicates that the codon is chosen randomly, RSCU >1 indicates that the codon is more frequent than expected and RSCU <1 indicates that the codon chosen is less frequent.

RSCU calculation formulae: $RSCU = (g_{ij} \times n_j) / (\sum_i^m g_{ij})$ (Sharp and Li 1986)

Where g_{ij} = observed number of codons for i^{th} and j^{th} amino acid that has n_j kinds of synonymous codon.

The effective number of codons (ENC or Nc) was calculated in Codon W 1.4.2. The ENC is used to quantify the codon usage bias of each gene (Wright 1990).

ENC calculation formulae: $ENC = 2 + S + [29 / \{S^2 + (1 - S)^2\}]$

Where S represents the value of G+C at the third position (GC3) (Wright 1990).

The ENC values were correlated to the percentage of GC3s. When codon usage is biased only one for each amino acid is used and the ENC is 20. If the ENC value is 61 then there is no bias in codon usage and all synonymous codons are equally used. The aliphatic index (AI) refers to the relative volume of a protein that is occupied by aliphatic side chains

(Alanine, Isoleucine, Valine and Leucine) and contributes to the increased thermostability observed for globular proteins (Ikai 1980).

AI calculation formulae: $AI = X(A) + aX(V) + [bX(I) + X(L)]$

Where X(A), X(V), X(I) and X(L) are mole percent of alanine, valine, isoleucine and leucine, respectively. The coefficients a, b are relative volumes of V (a = 2.9) and L/I side chains (b = 3.9) relative to alanine side chains. The grand average hydropathy (GRAVY) value determines the hydrophobic and hydrophilic properties of proteins. GRAVY is calculated as the sum of the hydropathy values of all the amino acids that are present in a protein divided by the total number of residues (Kyte and Doolittle 1982). The negative values indicate non polar proteins and positive values indicate polar proteins. Correspondence analysis (COA) enables the identification of the major trends in the variation of data and distribution of genes along continuous axes in accordance with these trends. For the major trend to influence codon usage variation in genes the variability should be more than 10% (Greenacre 1984). The COA of the 53 sequences was performed in Codon W 1.4.2. The correspondence analysis was used to study the variations in codon usage. The major trends studied were the axes of relative inertia. All the statistical analysis was carried out using the statistical analysis software SPSS Statistics Version 20.0. Correlation analysis was performed using Pearson's correlation analysis method with statistical significance at p-value ≤ 0.05 and 0.01.

The most used codons were U-ended, C-ended and A-ended, whereas, interestingly no G-ended codons were used as preferential codons. The GC3s and GC content of the gene are displayed in table 1. The RSCU values were calculated to study the variation in codon usage bias in IBV S gene. The details results of RSCU in 53 IBV S genes are displayed in table 2. The average GC3s% content of IBV S gene was found to be 24.4415% whereas, GC% was

found to be 36.33%. The mean GC3 is greater than that of the GC in the isolates of IBV. These results are conclusive of the fact that IBV S gene is not GC rich gene and most codons are A- and U-ended.

The Nc values range from 20 to 61. The higher the extent of codon preference, the smaller is the Nc value. A gene exhibiting no codon bias has Nc equal to 61. Conversely, in a highly biased gene one codon is used for each amino acid thus Nc equals 20. Our data of IBV S genes showed that the Nc value varied from 40.15 to 58.98, with a mean of 44.2413 with a standard deviation of 3.6085, which indicated that the codon usage bias in IBV S gene was small and having less variation.

The COA was performed using Codon W 1.4.2 to investigate codon usage variation in the 53 isolates of IBV S protein gene selected for this study. The position of each S gene defined by the first and second axes generated by COA on RSCU values as depicted in Figure 1. The plot value of first and second axes was made. The first principal axis accounted for 34.503% of the total variation, the second, third and fourth axis accounted for 22.245%, 21.974% and 18.278%, respectively. Therefore, the first axis had a major impact on the total variation in synonymous codon usage in comparison to the second axis (Figure 1). Moreover, a greater G + C compositional constraint was recorded at the third position (Table 3). This indicates that most of the codon usage bias among different IBV S genes is related to the pattern of base composition at the third position also deduced by the ENC plot (Figure 1).

The codon chosen by a mutation bias of G+C lie in a curve of predicted values, if the values lie above the curve then codons are assumed to be chosen by natural selection (Wright 1990; Zhong et al. 2007). Our results showed that all the points were found below the expected curve, which suggests that the codon bias in the 53 IBV S protein genes is affected by mutation (Figure 2). Therefore, the compositional constraint is one of the determinants of

the variation in synonymous codon usage in IBV S genes. The patterns of base composition were most likely the results of mutational bias, and not natural selection. The main factors accounting for codon usage variation in genes are thought to be mutational pressure and translational selection (Jenkins and Holmes 2003). To investigate the role of mutational bias as a determinant of codon usage variation in IBV S genes, correlation analysis was employed to correlate first two axis of COA with codon usage indices. No correlation was observed between the two axes, GRAVY, aromaticity, GC3s and AI (Figure 2).

Aromaticity and hydrophobicity determines the codon usage variation (Lobry and Gautier 1994). In order to investigate whether selection pressure contributes to the codon usage variation among S genes, a correlation analysis to evaluate whether aromaticity (AROMO) and GRAVY values were related to the first two axes of the COA and codon usage indices was performed. Similarly, we have also performed a correlation analysis of AI with various codon usage indices to study the role of AI in codon bias (Table 3). Our correlation analysis results showed that there was significant correlation observed between AROMO and G+C content of the IBV S gene (Table 3). GRAVY was found to be positively correlated with GC3s ($r = 0.700, P < 0.001$) and with ENC (Table 3). There was no significant correlation observed between GRAVY and GC1, GC2, and GC. Nc was found to be positively correlated with GC3s ($r = 0.955, P < 0.001$), AI ($r = 0.288, P < 0.05$), GC ($r = 0.399, P < 0.05$), GRAVY ($r = 0.670, P < 0.001$) and AROMO ($r = 0.562, P < 0.001$). GC3s were found to be positively correlated with GC ($r = 0.495, P < 0.001$), GRAVY ($r = 0.700, P < 0.001$) and AROMO ($r = 0.697, P < 0.001$). GC was found to be positively correlated with NC ($r = 0.399, P < 0.05$) GC3s ($r = 0.495, P < 0.001$) and AROMO ($r = 0.724, P < 0.001$).

Interestingly, none of Nc, GC3s, AROMO, GRAVY and AI were found to be correlated with axis 1 or axis 2 of COA, indicating that the codon usage bias IBV S gene is entirely due to mutational pressure. However, selection pressure for the codon usage pattern in IBV genome cannot be ruled out. The results indicated that the aromaticity, degree of hydrophobicity and aliphatic index have no association with the codon usage variation in IBV S genes. The significance of these conclusions remains to be determined. The study will help us to understand the variation in S protein gene and the outbreak of IBV in poultry. Further studies on other genes and its correlation with S would give a better insight of IBV genome and its codon usage.

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Conflict of interest:

The authors declare no conflict of interest.

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Figure legends

Figure 1: N_c of codons used plotted against the GC3s IBV S gene. The continuous curve as seen in the plot represents the relationship between GC3s and the N_c in the absence of selection. All the values lie below the expected curve

Figure 2: Scatter plot of value of the first and second axis of each IBV S gene in COA. The first axis accounts for 34.503% of all variation and the second axis accounts for 25.245% of total variation.

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Figure 1:

Figure 2:

TABLES:**Table 1.** List of infectious bronchitis virus spike gene used for analysis of synonymous codon usage in the study. Bold letters indicates the lower and higher value of Nc.

S.No	Accession Number	Mononucleotide frequencies(%)				Nc	GC3s(%)	GC(%)
		T3s	C3s	A3s	G3s			
1	A12747	39.36	29.82	38.99	19.49	51.83	39	38.90
2	AY31930	65.33	12.05	30.81	13.83	40.15	19.9	35.60
3	AY338732	31.75	18.74	39.71	32.55	52.67	39.7	37.20
4	AY702975	62.02	10.92	34.59	16.98	42.08	20.7	35.40
5	AY761141	61.61	12.76	32.89	16.38	41.74	22	36.10
6	AY189157	60.28	13.10	32.69	18.22	43.66	23.5	36.40
7	AY692454	61.38	12.44	33.61	16.13	42.16	21.6	36.20
8	AY702085	61.47	12.98	32.93	16.25	42.45	22.1	36.20
9	DQ068701	58.73	13.15	33.65	18.47	44.75	23.9	37.00
10	DQ490205	59.68	13.17	34.75	18.02	43.18	23.2	36.10
11	DQ490206	38.86	31.09	39.97	18.40	50.78	39.3	39.40
12	DQ490209	60.41	12.39	34.54	17.48	43.64	22.4	36.30
13	DQ490210	61.49	12.40	33.77	16.38	42.51	21.7	36.20
14	DQ490211	61.03	12.59	33.89	16.73	43.08	22.1	36.20
15	DQ490212	52.87	15.11	38.79	20.13	47.67	26.2	36.60
16	DQ490213	61.43	12.49	33.73	16.45	42.57	21.8	36.10
17	DQ490214	59.53	14.40	33.29	17.38	44.18	24	36.40
18	DQ490215	59.59	13.50	34.70	17.84	43.11	23.4	36.20
19	DQ490216	60.41	13.27	34.18	17.22	43.08	22.9	36.20
20	DQ490217	60.16	13.25	34.70	17.04	42.59	22.7	36.00
21	DQ490218	61.49	12.46	33.41	16.51	42.7	21.9	36.20
22	DQ490219	55.31	15.57	38.15	17.05	47.13	24.5	36.30
23	DQ490221	61.29	12.40	34.05	16.38	42.6	21.7	36.10
24	DQ646404	59.48	13.04	35.19	16.07	43.21	22.1	36.10
25	DQ646406	60.18	12.54	35.18	16.11	42.49	21.6	36.00

26	DQ490207	55.53	15.49	38.24	17.08	46.58	24.4	36.10
27	DQ490208	60.36	12.68	33.17	17.98	43.56	23.1	36.50
28	DQ490220	55.31	15.57	37.91	17.32	47.33	24.7	36.30
29	EU822336	60.14	12.71	34.62	16.78	42.72	22.3	36.10
30	EU822337	59.76	12.13	34.20	18.08	43.09	22.8	36.50
31	EU822338	58.62	13.63	33.74	17.98	43.42	23.9	36.90
32	EU822339	60.34	12.37	33.98	17.44	43.24	22.4	36.20
33	EU822340	58.73	13.22	33.85	18.46	43.96	23.9	36.80
34	EU822341	61.63	12.79	32.93	16.36	41.9	22	36.10
35	FJ829873	61.93	11.98	33.17	16.67	41.94	21.5	36.10
36	FJ829875	62.01	12.06	33.37	16.13	41.81	21.3	36.00
37	FJ829878	62.22	11.82	33.37	16.06	42.2	21	35.90
38	FJ829880	61.85	12.35	33.01	16.03	42.15	21.5	36.20
39	FJ829881	62.02	11.92	33.33	16.17	41.94	21.2	36.00
40	FJ829882	60.76	12.41	33.91	17.05	42.9	22.1	36.00
41	FJ829883	61.94	12.35	33.54	15.83	42.22	21.2	35.80
42	FJ829872	60.47	12.44	32.93	18.34	43.7	23	36.30
43	FJ829874	61.54	12.75	33.01	16.26	42.09	21.9	36.10
44	FJ829877	62.12	12.02	33.41	15.83	42.28	21	35.90
45	FJ829879	61.62	12.22	33.21	16.42	42.3	21.6	36.10
46	GQ229232	59.41	13.97	34.42	16.69	43.36	23.2	36.40
47	GU386375	61.74	12.85	33.33	16.11	41.7	21.8	36.00
48	JF732918	57.00	12.98	37.45	19.73	45.57	24	35.70
49	KF188436	61.63	12.79	32.53	16.73	41.93	22.3	36.30
50	M21515	42.86	12.24	26.09	50.00	58.98	44.1	33.30
51	U49858	39.91	30.16	38.36	20.72	51.96	39.6	38.70
52	U04739.1	39.91	29.99	38.42	20.57	52.01	39.4	38.70
53	X87238.1	60.60	12.06	37.21	14.84	41.94	20.3	35.00

Table 2. Infectious bronchitis virus spike gene specific synonymous codon usage. The RSCU is cumulative relative synonymous codon usage. The overall values depict the codon usage for the S1 gene for all 53 isolates.

Amino acid	Codon	Codon Repeat	RSCU	Codons predominantly used in S protein gene
Phe	UUU	2880	1.6970	UUU
	UUC	536	0.3030	
Leu	UUA	1440	1.7136	UUA
	UUG	1110	1.3679	
Tyr	UAU	2272	1.3830	UAU
	UAC	1013	0.6170	
Leu	CUU	1365	1.6255	CUU
	CUC	249	0.2908	
	CUA	529	0.6089	
	CUG	322	0.3947	
His	CAU	563	1.2687	CAU
	CAC	333	0.7313	
Gln	CAA	1642	1.2013	CAA
	CAG	1118	0.7987	
Ile	AUU	2089	1.6368	AUU
	AUC	382	0.3192	
	AUA	1341	1.0434	
Asn	AAU	3450	1.6525	AAU
	AAC	643	0.3474	
Lys	AAA	1283	1.0670	AAA
	AAG	1103	0.9330	
Val	GUU	2367	2.0928	GUU
	GUC	379	0.3504	
	GUA	973	0.8619	
	GUG	768	0.6942	
Asp	GAU	1522	1.2947	GAU
	GAC	711	0.76675	
Glu	GAA	1277	1.2666	GAA
	GAG	691	0.7334	
Ser	UCU	2000	2.0045	UCU
	UCC	289	0.3225	
	UCA	1166	1.3385	
	UCG	134	0.1526	
Cys	UGU	1558	1.5326	UGC
	UGC	536	0.4674	
Pro	CCU	1138	2.1166	CCU
	CCC	149	0.2975	
	CCA	697	1.2860	
	CCG	167	0.3004	
Arg	CGU	618	2.0240	CGU
	CGC	44	0.1204	
	CGA	96	0.2779	
	CGG	46	0.1183	
Thr	ACU	2383	2.1072	ACU
	ACC	425	0.4077	
	ACA	1306	1.1951	
	ACG	297	0.2906	
Ser	AGU	1588	1.8217	AGU
	AGC	292	0.3609	
Arg	AGA	785	2.4515	AGA
	AGG	316	0.8942	
Ala	GCU	1527	1.6687	GCU
	GCC	503	0.5809	
	GCA	1228	1.4089	
	GCG	230	0.2670	
Gly	GGU	2352	2.2142	GGU
	GGC	701	0.7198	
	GGA	659	0.6591	
	GGG	310	0.4006	

Table 3. Summary of correlation analysis between the first two axes in COA, GC3s, GRAVY, aromaticity and aliphatic Index (AI) in the selected complete spike gene sequences for infectious bronchitis virus isolates. P -value ≤ 0.05 ; P -value ≤ 0.01 were used for the correlation analysis

		Nc	GC3s	AI	GC	Gravy	Aromo
Axis 1	r	.010	.034	-.119	.058	.070	.044
	p	.942	.811	.397	.681	.619	.755
Axis 2	r	-.040	.001	-.021	.097	.043	.065
	p	.776	.991	.884	.491	.762	.645

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