ROLE OF RELATIVE CODON USAGE (RSCU) ON INFECTIVITY AND VIRULENCE OF FOWL ADENOVIRUS ISOLATED FROM INFECTED IRAQI POULTRY

Aya .R. Abdulla[1] **Rebah .N. Jabbar[2]** **Aida .B. Allawe[1]**
Researcher **Prof.** **Prof.**
[1] Dep. Micro, Coll. Vet Med, University of Baghdad
[2] Biotech Rese Cen, Al-Nahrain University, Baghdad
Email:tuta.fh2017@gmail.com

ABSTRACT

This study was aimed to investigate the role of codon usage on infectivity and virulence of fowl adenovirus. The PCR amplicon sequence revealed that loop1 has a hypervariable region and is adjacent to loop2 on Hexon A segment, while Hexon B segment has a less variable region. The fiber gene is similar among isolates, with differences associated with the virus. Most belong to fowl adenovirus-4, with others in type 8b and E. The study revealed increased effective nucleotide codons in isolates 5, 33, 44, and 15 in Fiber gene, Loop2, and Loop1genes. The study analyzed the impact of mutation on codon usage, hypervariable regions 1-4, and the effective number of codons by determining high and low effective usage. Fowl adenovirus has hypervariable regions that extend at a specific area along HexA segment. The study found stable regions in viruses with variable nucleotides, indicating their evolutionary capability and abundance of amino acids Alanine, Glycine, Proline, Arginine, and Serine, as detected by relative codon usage calculation. The high usage of these codons may result in mutational pressure, potentially aiding in understanding the infectivity and pathogenicity of Fowl adenoviruses through natural selection.

Keywords: avian viruses, fiber gene, hexon gene, viral genome analysis.

دور استعمال الكودون النسبي (RSCU) في العدوى والضراوة للفيروس الغدي للطيور المعزول من الدواجن العراقية المصابة

[1] اية رياض عبدالله **[2]رباح نجاح جبار** **[1]عائدة برع علاوي**
باحث **أستاذ** **أستاذ**
[1]فرع الأحياء المجهرية– كلية الطب البيطري – جامعة بغداد
[2]مركز بحوث التكنولوجيا الحيوية، جامعة النهرين، بغداد، العراق

المستخلص

تهدف هذه الدراسة إلى التحقق من دور استعمال الكودون في العدوى والضراوة للفيروس الغدي للطيور. كشف تسلسل تفاعل البلمرة للأمبليكون أن الحلقة 1 تحتوي على منطقة شديدة التغير وهي مجاورة للحلقة 2 على مقطع Hexon A، بينما يحتوي مقطع Hexon B على منطقة أقل تغيرًا. ويتشابه جين الألياف بين العزلات، مع وجود اختلافات مرتبطة بالفيروس. ينتمي معظمها إلى فيروسات الطيور الغدية–4، مع وجود فيروسات أخرى من النوع b8 وE. كشفت الدراسة عن زيادة في كودونات النيوكليوتيدات الفعالة في العزلات 5 و 33 و 44 و 15 في جينات الألياف و الحلقة1 والحلقة2. قامت الدراسة بتحليل تأثير الطفرة على استعمال الكودونات، والمناطق شديدة التغير 1–4، والعدد الفعال للكودونات من خلال تحديد الاستخدام الفعال العالي والمنخفض. يحتوي فيروس الطيور الغدي على مناطق شديدة التغير تمتد في منطقة معينة على طول مقطع HexA. وجدت الدراسة مناطق مستقرة في الفيروسات ذات النيوكليوتيدات المتغيرة، مما يشير إلى قدرتها التطورية ووفرة الأحماض الأمينية ألالنين، الجلايسين، البرولين، الأرجينين، والسيرين، كما تم الكشف عنها عن طريق حساب استعمال الكودون النسبي. قد يؤدي الاستعمال العالي لهذه الكودونات إلى ضغط طفري، مما قد يساعد في فهم العدوى والاصابة بفيروسات الطيور الغدية من خلال الانتقاء الطبيعي.

الكلمات المفتاحية: تحليل الجينوم الفيروسي, جين الالياف، جين الهيكسون, فيروسات الطيور.

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INTRODUCTION

A significant contributor to the economic development of any nation is the production of high-quality chicken meat protein for human consumption by broiler farms, which also provide a number of societies with a good source of profitability (19). Adenovirus which is a part of adenoviridae is one of the causative agents in many of diseases regardless of the site of infection, and symptoms. Viruses consist essentially of genetic material and structural proteins that encapsulate it (10).The viral particle contains a linear DNA, double-stranded DNA molecule with size ranging from 25–46 kbp. Adenoviruses isolated from poultry are mainly fowl adenoviruses (FAdVs) with five species FAdV A – E (5,6). FAdV is an etiological agent of disease such as inclusion body hepatitis (IBH) (13). This disease primarily affects broilers up to 35 days old, while it has occasionally been seen in all age groups (15). Potentially the fastest and most accurate approaches for detection and identification are those based on nucleic acid amplification (1,27). Sensitive diagnostic techniques like sequencing and phylogenetic analysis were necessary for accurate detection of the presence of a certain species in particular regions or nations. (11,14,18). When molecular analysis of genes was performed, there were major structural proteins constructed with the antigenic properties of the capsid. These were identified as Hexon and Fiber genes products (25). The genome assembly showed the presence of 1-8 sequences with many transcriptional units identified by their open reading frames (ORFs). Even with all genes are identified, not all of their role and function are known (8), but when RSCU was taken into concern, the measurement of divergence can be calculated by gene expression, evolution, and synonymous codons usage pattern that was reported by other researchers (3). The RSCU comes with different values up to one as the point of reference. When it is higher than 1, it means a high frequency of codon usage than expected, while less than 1, means the RSCU is with less frequency than expected (24). The pressure of mutation and RSCU relation was established by studying triplets compositions at the 3rd codon position (17). Niczyporuk reported the RSCU values for type adenoviruses regardless of their families. This study was designed to estimate the effect of RSCU of virulence genes in different genotypes of fowl adenovirus (FAdV) and the outcome of infectivity and virulence in these novel newly identified strains.

MATERIALS AND METHODS

FAdV Sample collection

The FAdV samples (livers) were collected from suspected infected chickens from 3provinces, Karbalaa (32.5 43.8), Diyala (45,0 34,0) and Tikrit (34.5 43.65), were reported to have an outbreak of this disease distributed as follow: 25 sample from Karbalaa, 10 samples from Diyala, and 15 samples from Tikrit. Liver organ samples were transferred aseptically in cool box and kept under – 20℃ until processing.

DNA isolation from liver organs

DNA was isolated from infected livers using Favorgene DNA isolation kit batch no. Fav20061 according to the manufacturer instruction. A 50µl DNA sample was obtained from each sample with purity of 1.8 as measured spectrophotometrically and freezed under – 20℃ for PCR amplification.

Primers

Different primers were used during this work. Each primer is responsible for amplification of different gene of FAdV genome as shown in Table (1.)

Table 1 Primer sequence for genes of the study.

Primer sequence 5' – 3'	Amplified gene	Reference
F –AATGTCACNACCGARAAGGC R- CBGCBTRCATGTACTGGATA	L1 loop	(4)
F-AACTTCGACCCCATGTCGCGTCAGG R-TGGCGAAAGGCGTACGGAAGTAAGC	L2 loop	(22)
F-GGTCTACCCCTTTTGGCTCC R-GCGTCGTAGATGAAGGGAGG	Fiber	(23)
F- CGTCTAGGTTCGCACCGCCATGGC R- CATCTGGTCGATGGACCAACGCGCACC	Hexon A	(21)
F- CATCGACCAGATGGACAACGTCAACCCCTTCAAC R-TTACACGGCGTTGCCTGTGGCG	Hexon B	(21)
Real – time PCR F-CAGTTCATTTCCGCCACC R-GCAGCCGTTGAGCCTTTT		(22)

PCR amplification conditions

To obtain optimum amplification of L1 segment, the following program was used for this purpose: initial denaturation step at 95℃ for 5min/ and amplification for 35 cycles for denaturation at 95℃ for 10s/ anneling at 65℃ for 45s/ and extension step72 ˚C for 2 min and final extension step at 72℃ for 10min., while L2 loop was amplified using the same reaction condition but with 55℃ as annealing temperature for 1min. Additionally, HexonA (HexA) segment and Hexon B (HexB) segment were amplified using the same conditions with different annealing temperatures which were 66℃ (for fragment A) and 57℃ (for fragment B) last for 30s with extension time at 72 °C for 80s; ended with final extension at 72 °C for 5 min.

Gel electrophoresis

Resulted PCR amplicons were resolved by electrophoresis in 1.5% agarose with field strength for 1 hour. Bands were stained with EtBr for 30 min. and visualized by UV transilluminator MSE-280. The resulting amplicons from genes subjected to amplification were sent to Macrogene, Korea for sequencing using the Sanger sequencing method.

Data analysis

DNA sequence obtained from PCR amplification were analyzed using NCBI blast algorithm available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastnandPAGE_TYPE=BlastSearchandLINK_LOC=blasthome, Uniport blast algorithm available at: https://www.uniprot.org/blast. Tools for building a phylogenetic tree, and similarity matrix were also available at: https://www.uniprot.org/. for calculating RSCU, DNasp version 6 package was employed after generating appropriate alignment files.

NCBI registration

DNA sequences obtained from each gene were submitted to NCBI Bankit, and are available under ID no. shows in Table (2.)

Table 2. Sequence registration of analyzed genes from FAdV isolates under study

ID	Date of submission	Submitted record	Title
2659945	02 Jan 2023 06:58:43	Download File (*.gz)	Fiber gene
2655527	16 Dec 2022 10:22:01	Download File (*.gz)	L1 loop
2655518	16 Dec 2022 10:06:06	Download File (*.gz)	L2 loop
2655516	16 Dec 2022 09:57:34	Download File (*.gz)	Fowl adenovirus HexonB
2655514	16 Dec 2022 09:48:07	Download File (*.gz)	Fowl adenovirus Hexon A

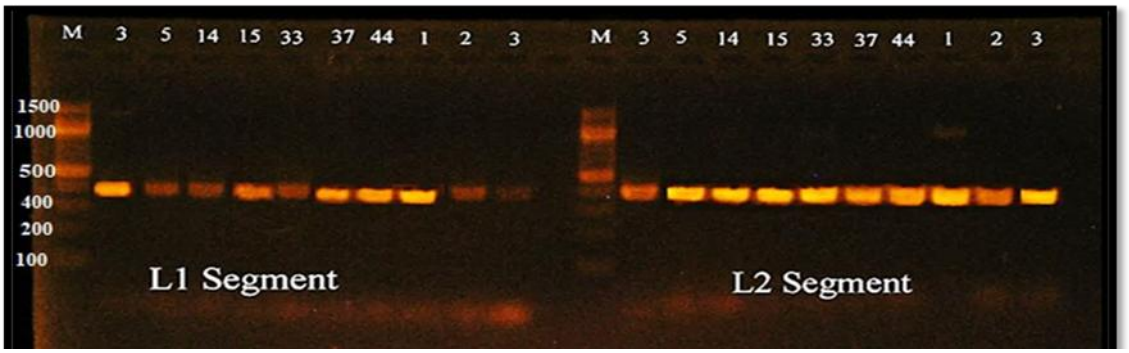
RESULTS AND DISCUSION

In this study, 50 liver samples suspected to be infected with FAdV were obtained from different chicken farms and subjected to analysis. molecular confirmation was conducted by PCR for identification fowl adenovirus by specific primers of L1 gene and L2 gene that are considered as key feature genes responsible for encoding viral capsid protein, generated bands at the location of 400bp for each (L1, L2) on an agarose gel. As shown in Figure(1), the result showed that

only 30 out of 50 isolates were positive for fowl adenovirus, this results agreement with result of (12).

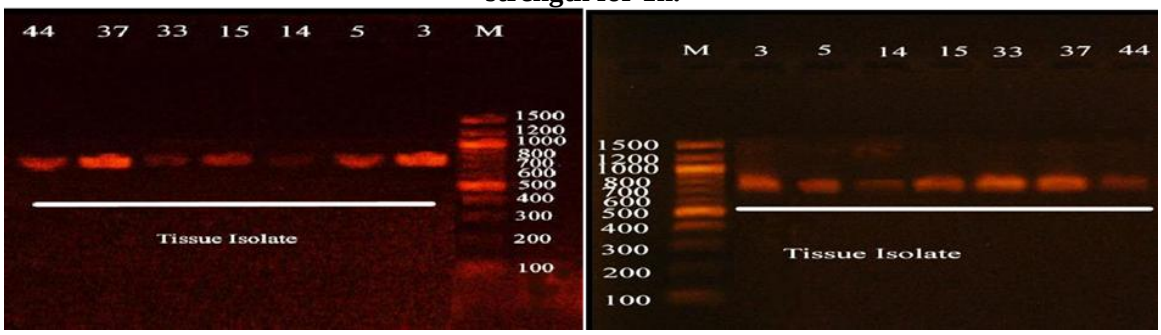
Analysis of L1, L2, Hexon A, Hexon B and Fiber gene (F gene) genes

Detection of virulence genes: The hexon gene contains hypervariable regions (HVR) that in the external part of the virion resembled with fragments L1, L2, and L4 loops. Regions HRVs 1 – 7 were identified, four of them are positioned within Loop 1 (L1), this result agreement with result of (26,12,28). The Hexon genome segment from confirmed infection with FAdV were amplified by PCR using specific primer and sequenced for data analysis. Figure (2) shows the amplification results of HexA (800bp), HexB (500bp) and Fiber (400bp).

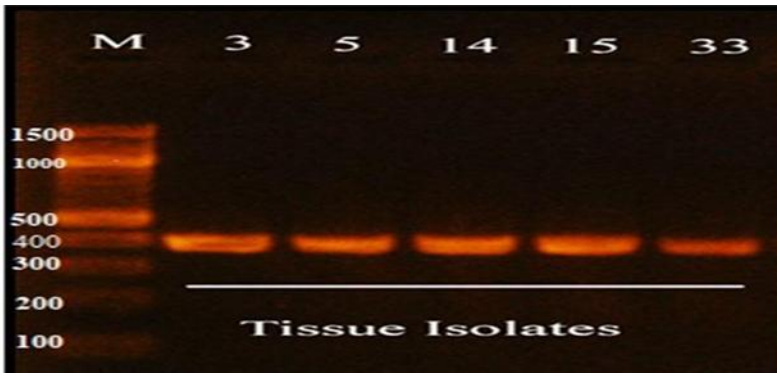


A B

Figure 1. PCR specific amplification of L1 segment (400bp)(A), L2 segment (400bp) (B), M molecular weight marker(100-1500bp). Electrophoresis was performed in 1.5% agarose gel at 8 v/cm field strength for 1h.



A B



C

Figure 2. PCR specific amplification of HexA segment (800bp)(A), HexB segment (500bp) (B) and Fiber gene (400bp) (C), M molecular weight marker(100-1500bp). Electrophoresis was performed in 1.5% agarose gel at 8 v/cm field strength for 1h.

Genotyping of detected viruses: All virus samples were genotyped depending on blast results of DNA sequences for hexon gene obtained from PCR amplification. The main criterion for genotype is highly dependent upon e – value obtained from blast that been

listed in Table (3.). The result showed presence of multiple genotypes as FAdV-4, FAdV-8b and E, this result agreement with (12) which found FAdV-4,FAdV-8b, FAdV-8a and E.

Table 3. Genotyping of viral samples depending on Hexon gene.

Accession no. match	Isolate no.	Gene	Organism	Query coverage	e-value
A0A142CQY2	3	Hexon	Fowl aviadenovirus 4	2 – 421	1.1 E-87
A0A142CQY2	5	Hexon	Fowl aviadenovirus 4	4 – 441	3.3E-95
A0A142CQY2	14	Hexon	Fowl aviadenovirus 4	3 – 443	2.1E-93
A0A142CQY2	15	Hexon	Fowl aviadenovirus 4	2 – 448	5.2E-90
A0A142CQY2	33	Hexon	Fowl aviadenovirus 4	2 – 448	6.4E-85
A0A142CQY2	37	Hexon	Fowl adenovirus 4	3 – 449	1.0E-88
E9KLA6	44	Hexon	Fowl aviadenovirus E	3 – 452	3.5E-80

Analysis of relative synonymous codon usage (RSCU): The RSCU of studied genes was analyze using DNaSp version 6 to generate the table in which each appear during genome activity of the virus. Results are shown in Figure (3.)

FAdV-4

Scaled Chi-squared: 0.101

UUU-F	10	(0.95)	UCU-S	5	(0.91)	UAU-Y	15	(1.20)	UGU-C	2	(0.57)
UUC-F	11	(1.05)	UCC-S	8	(1.45)	UAC-Y	10	(0.80)	UGC-C	5	(1.43)
UUA-L	9	(1.17)	UCA-S	7	(1.27)	UAA-*	13	(1.70)	UGA-*	8	(1.04)
UUG-L	6	(0.78)	UCG-S	3	(0.55)	UAG-*	2	(0.26)	UGG-W	8	(1.00)
CUU-L	8	(1.04)	CCU-P	8	(0.89)	CAU-H	3	(0.75)	CGU-R	2	(0.33)
CUC-L	9	(1.17)	CCC-P	14	(1.56)	CAC-H	5	(1.25)	CGC-R	10	(1.67)
CUA-L	7	(0.91)	CCA-P	8	(0.89)	CAA-Q	11	(1.22)	CGA-R	5	(0.83)
CUG-L	7	(0.91)	CCG-P	6	(0.67)	CAG-Q	7	(0.78)	CGG-R	5	(0.83)
AUU-I	10	(1.07)	ACU-T	7	(0.64)	AAU-N	11	(0.71)	AGU-S	5	(0.91)
AUC-I	4	(0.43)	ACC-T	12	(1.09)	AAC-N	20	(1.29)	AGC-S	5	(0.91)
AUA-I	14	(1.50)	ACA-T	15	(1.36)	AAA-K	15	(1.20)	AGA-R	9	(1.50)
AUG-M	3	(1.00)	ACG-T	10	(0.91)	AAG-K	10	(0.80)	AGG-R	5	(0.83)
GUU-V	13	(2.08)	GCU-A	5	(0.77)	GAU-D	7	(0.82)	GGU-G	4	(0.76)
GUC-V	1	(0.16)	GCC-A	6	(0.92)	GAC-D	10	(1.18)	GGC-G	4	(0.76)
GUA-V	4	(0.64)	GCA-A	8	(1.23)	GAA-E	11	(1.22)	GGA-G	7	(1.33)
GUG-V	7	(1.12)	GCG-A	7	(1.08)	GAG-E	7	(0.78)	GGG-G	6	(1.14)

FAdV-E

Scaled Chi-squared: 0.180

UUU-F	7	(1.08)	UCU-S	3	(0.64)	UAU-Y	5	(1.25)	UGU-C	2	(1.00)
UUC-F	6	(0.92)	UCC-S	8	(1.71)	UAC-Y	3	(0.75)	UGC-C	2	(1.00)
UUA-L	1	(0.13)	UCA-S	4	(0.86)	UAA-*	1	(0.75)	UGA-*	1	(0.75)
UUG-L	8	(1.02)	UCG-S	3	(0.64)	UAG-*	2	(1.50)	UGG-W	7	(1.00)
CUU-L	6	(0.77)	CCU-P	5	(0.80)	CAU-H	3	(1.50)	CGU-R	2	(0.63)
CUC-L	18	(2.30)	CCC-P	11	(1.76)	CAC-H	1	(0.50)	CGC-R	2	(0.63)
CUA-L	2	(0.26)	CCA-P	4	(0.64)	CAA-Q	13	(1.04)	CGA-R	1	(0.32)
CUG-L	12	(1.53)	CCG-P	5	(0.80)	CAG-Q	12	(0.96)	CGG-R	2	(0.63)
AUU-I	5	(0.83)	ACU-T	8	(0.68)	AAU-N	10	(0.74)	AGU-S	5	(1.07)
AUC-I	9	(1.50)	ACC-T	17	(1.45)	AAC-N	17	(1.26)	AGC-S	5	(1.07)
AUA-I	4	(0.67)	ACA-T	11	(0.94)	AAA-K	15	(1.15)	AGA-R	5	(1.58)
AUG-M	4	(1.00)	ACG-T	11	(0.94)	AAG-K	11	(0.85)	AGG-R	7	(2.21)
GUU-V	8	(1.03)	GCU-A	5	(0.57)	GAU-D	9	(0.67)	GGU-G	6	(0.67)
GUC-V	11	(1.42)	GCC-A	15	(1.71)	GAC-D	18	(1.33)	GGC-G	9	(1.00)
GUA-V	2	(0.26)	GCA-A	8	(0.91)	GAA-E	13	(1.04)	GGA-G	17	(1.89)
GUG-V	10	(1.29)	GCG-A	7	(0.80)	GAG-E	12	(0.96)	GGG-G	4	(0.44)

Figure 3. The distribution of RSCU for FAdVs strains under study. Synonymous codons are indicated in colors. The intensity of the color indicates the preferability of an amino acid a particular position amongst species. Codons taking the same color with redundancy, are preferable during translation, while codons with less color redundancy are less translated.

For all data input the Number of sequences were 45 and Number of sequences used: 45 with Selected region: 1-2733 and Number of sites: 2733. Total number of sites (excluding sites with gaps / missing data): 0. The parameters used to analyze the genetic code were as follow: the genetic code accommodated was the universal type that encodes for protein, and non – coding regions (intronic and flanking regions) were found to be 0. Additionally, Average number of analyzed codons: 317.978 and No. Codons excluding those of amino acids coded by a single codon: 299.511. The effective no. of codons (ENC) was calculated to be 56.989, and codon bias index (CBI) was 0.251. The Scaled Chi-square, SChi2 was calculated to be 0.241 computed without Yates' correction. **G+C content:** Both nucleotide positions (2nd and 3rd) were determined as follow: the second codon position G+C2 was calculated to be 0.540 with Average number of sites: 310.33, while G+C content at (synonymous) third codon positions, G+C3s was calculated to be 0.583 with Average number of sites of 299.51. the coding positions resembled with G+Cc was found to be 0.564 considering an average number of sites to be: 931.00. All Sites/Codons with alignment gaps were excluded in single DNA sequences. There was a degeneracy of the genetic code detected in several groups of synonymous codons, whereas others were preferred compared to others. This was termed as codon bias. It is associated with different factors resembled with natural selection, RNA structure, gene length, and mutational pressure (17). The codon usage is a reliable technique to understand at molecular level the process of evolution (22). During data analysis in this study, the frequency of nucleotide in coding triplets depending on GC content and its differences according to the type or species of examined sample.

The value calculated ranged to be 54.2% for both FAdV-4 and FAdV-E that are close to what was reported by (5). It is reported by (13), that genes with high transcriptional level may contain high GC ratio. The deviation of RSCU is based mainly on codon preference when the same codon is more frequently used during translation process than others synonymous once in high expression genes that is referred to as the optimal or preferable codon (3). From evolutionary point of view, it may be considered as an adaptation to optimize gene expression, the percentage of GC content, reconstruct genome structure, and or the frequency of recombination. Moreover, the codon usage for genotypes FAdV-4, and FAdV – E was calculated to establish and evaluated if the preference codon located in hypervariable regions (HVRs 1– 4) located within hexon gene appeared or not. In previous reports (20) RSCU for FAdV-4 and E was estimated. Within Hex A gene, the presence of HVRs-1-4 within L1 segment is a clear indicator of variability that was confirmed by other researcher (13). Amino acid sequences obtained after codon translation were aligned for both genotypes which indicated a confirmed. location of HVR 1-4 to be located in L1. Results showed high variability in the amino acids sequence of FAdV-4 and FAdV-E at HVR 1-4 with the highest divergence compared to the consensus sequence. However, amino acid substitution may not inflect an effect on protein structure and function since most of these substitutions maybe with the same size, charge, and hydrophobic properties that represent amino acids with conserved function. Such fact can be explained depending on protein structure since it may pose the property of self-structure and function with different amino acids sequences encoded by highly different nucleotide sequences. We found 61 amino acids of 177 (36.2%) in L1 were identical in all examined sequences considered as consensus sequences. We can presume that everyone may have equal frequency when it is used, but when studied during this research, with several possibilities, only one codon is preferred that dominates in genes with high expression (26). Optimal codons lead to accurate translation which with crucial importance regarding proteins synthesized in high amounts (2). which means that may have an impact on viral evolution. When the mutational and evolution quantity is taken into concern during the analysis of the results, it is suggested by author (13). that mutations and codon variation in 1st and 2nd codon positions are the most important. These are active mutations that alter protein structure and function. In consequence, such changes affect viral pathogenicity. A principal component analysis also supported that most codons showed bias on G and C nucleotides at the 3rd position and dominant codons end with the same nucleotides. Other researchers (8). showed a positive and highly significant correlation between a few amino acids such as Val (V), lysine (L), and Ile (I) and gene expression that may be affect significantly that was confirmed in codon T/CT(N) existing in strains: FAdV-4/E codes for lysine Lys (L). our study showed that the percentage usage of GC pairs for different species or types was 54.2% for FAdV-4 and FAdV-E, with an average value of 56.3% which are similar to (5). We can estimate that genes with high transcription and expression level harbor high GC percentage pairs as suggested by (7). In previous report (9), a part of the major dominants is the GC pair at the 3rd position of the triplets with observed variation within the synonymous codon. Referring to a previous (20), the RSCU in L1 loop of Hexon gene may bear the most important mutational pressure at positions (1and 2) of the codon since when a change takes place at these positions it results in different amino acids that alter protein structure. Further analysis of codons in loop L1 showed differences in codon preference patterns and average number of codons among adenovirus strains indicating a diverse type.

CONCLUSION

As a conclusion, studying HVRs 1 – 4 RSCU can provide a supportive tool to study FAdVs evolution and develop a method for novel knowledge of the origin of HVRs with other FAdVs strains. Such analysis provides a clear picture of gene expression levels. Even more, when depending on analyzed data, it logically to conclude the presence of greater variation among species as they diverge affecting, eventually the codon usage. More indications

showed that variation in codon usage is type and species specific and determined by random mutation events during the evolutionary process. The present work is the codon analysis of a FAdV Loop L1 region of the hexon gene, and this is the first study conducted on RSCU on the FAdVs 4 /E in Iraq. The analysis indicated antigenic properties of the examined viruses, the presence of relative synonymous codon usage, and the presence of mutations. The effect of mutational pressure on the codon can be tested in future in order to understand its impact on FAdV pathogenicity.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

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