GENETIC DIVERSITY OF CHICKEN ANEMIA VIRUS FOLLOWING CELL CULTURE PASSAGING IN MSB-1 CELLS

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Summary. – It has been shown that a chicken anemia virus (CAV) isolates which had undergone 60 passages in MSB-1 cells (SMSC-1/P60, 3-1/P60) acquired 33–66 nucleotide substitutions at the coding region resulting in 13–16 amino acid changes as compared to the CAV isolates passaged only 5 times in MSB-1 cells (SMSC-1 and 3-1) (Chowdhury *et al*., *Arch. Virol.* **148**, 2437–2448, 2003). In this study we found that a low CAV (BL-5) and a high CAV passage (BL-5/P90) differed by only 15 nucleotide substitutions resulting in 11 amino acid changes. Phylogenetic analysis based on VP1 also revealed that both isolates were close to each other but not to other CAV isolates from Malaysia, namely SMSC-1 and 3-1.

Key words: Chicken anemia virus; virus passaging; nucleotide substitutions; amino acid substitutions; coding regions, nucleotide sequence; phylogenetic analysis

Introduction

Chicken anemia virus (CAV, the species *Chicken anemia virus*, the genus *Circovirus*, the family *Circoviridae*) is an economically important avian pathogen with a worldwide distribution (McNulty, 1991). The virus causes aplastic anemia and generalized lymphoid atrophy with a concomitant immunosuppression characterized by secondary bacterial or viral infections or vaccination failures (McNulty, 1991). The virus has a circular negative sense single-stranded DNA genome of 2.3 kb that contains three overlapping ORFs, namely VP1, VP2 and VP3 (Coombes and Crawford, 1996). VP1 is the capsid protein that plays an important role in virus spread and cell tropism (Renshaw *et al*., 1996). However, both VP1 and VP2 are essential for the formation of neutralizing antibodies in CAV-infected chickens (Koch *et al*., 1995), while VP3 is involved in the induction of apoptosis in chicken lymphoblastoid T cells (Noteborn *et al*., 1994) and in human malignant cell lines (Danen-Van Oorschot *et al*., 1997).

The sequence comparison of CAV isolates which had undergone low and high cell culture passage indicated that the VP2 and VP3 genes are relatively conserved while the VP1 gene is liable to changes (Chowdhury *et al*., 2003; Meehan *et al.*, 1997). It has also been shown that the pathogenicity of CAV can be significantly reduced by passaging in MSB-1 cells (Chowdhury *et al.*, 2003; Todd *et al.*, 1998). However, the attenuated CAV may regain its virulence by propagation in chickens (Todd *et al.*, 1995).

Recently, we have shown that CAV isolates, which had undergone many passages in MSB-1 cells acquired many nucleotide substitutions and grouped in separate clusters in the phylogenetic tree (Chowdhury *et l.*, 2003). The high passage isolates SMSC-1/P60 and 3-1/P60 (Chowdhury *et al.*, 2003) and the cloned isolate 10 (Meehan *et al.*, 1997) also shared two common amino acid substitutions V153A and C118R in VP2 and VP3, respectively. However, in this study we describe the genetic diversity of the isolate BL-5/P90 which has undergone 90 passage in MSB-1 cells as compared to that of the isolate BL-5 which has undergone only 5 passages in the same cells.

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Abbreviations: CAV = Chicken anemia virus; NS = non-synonymous; ORF = open reading frame; S = synonymous

Materials and Methods

Cell culture and virus isolates. The CAV BL-5 isolate underwent 5 passages in MSB-1 cells and was considered the low passage isolate, while the CAV BL-5/P90 isolate underwent 90 passages in the same cells and was considered the high passage isolate (Chowdhury *et al.*, 2000b).

PCR, cloning and sequencing. The CAV genome was amplified from infected MSB-1 cells by PCR (Chowdhury *et al*., 2002a). PCR products were purified by the Geneclean Kit (BIO101, USA) and cloned into the pCR® 2.1-TOPO vector (Invitrogen, USA). Recombinant clones were selected by PCR screening and confirmed by restriction analysis. Two independent clones for each of BL-5 and BL-5/P90 were subjected to sequencing in both directions using the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Perkin Elmer) in the ABI PRISM® 377 DNA Sequencer (Chowdhury *et al*., 2002a).

Sequence alignment and phylogenetic analysis. The isolates, Genbank Acc. Nos. and references for sequences used for sequence alignment and phylogenetic analysis were as follows: SMSC-1 (AF285882), 3-1 (AF390038), SMSC-1/P60 (AF390102), 3-1/P60 (AY040632) (Chowdhury *et al.*, 2003), Cux-1N (M55918) (Noteborn *et al.*, 1991), Cux-1M (M81223) (Meehan *et al.*, 1992), cloned isolate 10 (U66304) (Meehan *et al.*, 1997), CAU269/7 (AF227982) (Brown *et al.*, 2000), 704 (U65414) (Connor *et al.*, 1991), TR20 (AB027470), A2 (AB031296) (Yamaguchi *et al.*, 2001), 26P4 (D10068) (Claessens *et al.*, 1991), CIA-1 (L14767) (Renshaw *et al.*, 1996), BD-3 (AF3951141) (Islam *et al.*, 2002), 82-2 (D31965) (Kato *et al.*, 1995) and CAF475 (AF475908). The sequence data were analyzed using standard nucleotide BLAST 2.0.11 Program of National Institute of Biotechnology Information (NCBI) followed by the ClustalW Multiple Alignment Program under the BioEdit Software Package based on the nucleotide and amino acid sequences. Phylogenetic analysis was performed on the basis of amino acid sequences of VP1 using the neighbor-joining method of the ClustalX Program with 1,000 bootstrapping replicates. The sequences of BL-5 and BL-5/P90 were deposited at the GeneBank under Acc. Nos. AF527037 and AY150576, respectively.

Results and Discussion

Complete nucleotide sequences of the coding regions of the isolates BL-5 and BL-5/P90 were established from 2 independent plasmid clones for each isolate. No sequence changes were detected from the clones of both the isolates BL-5 and BL-5/P90. The length of the coding region in both the isolates was 1823 nucleotides that encoded three major ORFs consisting of 1350 bp, 651 bp and 366 bp for putative proteins of about 52 K (VP1), 24 K (VP2) and 14 K (VP3). All these ORFs were frame-shifted confirming earlier studies by Noteborn *et al.* (1991), Meehan et al. (1992), Kato *et al.* (1995), Renshaw *et al.* (1996), Meehan *et al.* (1997), Yamaguchi *et al.* (2001), and Chowdhury *et al.* (2003).

A comparison of similarity (%) of nucleotide and protein sequences of BL-5, BL-5/P90 and other CAV isolates is shown in Table 1. BL-5 showed a 99% similarity to BL-5/P90 with 15 substitutions (12 in VP1 and 3 in VP2). These substitutions resulted in 8 and 3 amino acid substitutions in VP1 and VP2, respectively. No substitutions were observed

Table 1. Comparison of homologies percentage and differences in nucleotide and amino acid sequences in VP1, VP2 and VP3 in BL-5, BL-5/P90 and other CAV isolates

CAV isolates	Origin	No. of passage in MSB-1	BL-5							BL-5/P90						
			Nucleotide				Amino acid			Nucleotide				Amino acid		
			%	VP1	VP2	VP3	VP1	VP2	VP3	%	VP1	VP2	VP3	VP1	VP2	VP3
BL-5/P90	Malaysia	90	99	12	3	0	8	3	0	–	–	–	–	–	–	–
SMSC-1	Malaysia	5	95	64	3	1	14	1	1	96	62	4	1	11	2	1
3-1	Malaysia	5	97	31	4	1	11	2	1	98	31	5	1	14	3	1
SMSC-1/ P60	Malaysia	60	97	27	4	2	11	2	2	98	31	5	2	11	3	2
3-1/P60	Malaysia	60	98	28	5	3	10	3	3	98	31	5	2	11	3	2
Cux-1M	Germany	ND	97	32	4	2	13	2	3	97	33	5	2	11	3	2
Cux-1N	Germany	ND	97	29	5	3	13	2	3	98	29	6	3	9	3	3
Cloned isolate 10	Germany	173	97	32	5	3	12	4	3	97	34	4	3	11	3	3
CAU269/7	Australia	ND	95	68	8	6	6	2	5	95	69	9	6	6	3	5
704	Australia	ND	95	62	3	2	12	2	2	96	60	5	2	11	3	2
A2	Japan	38	98	25	1	1	9	1	1	98	28	3	1	9	2	1
TR20	Japan	NP	95	63	4	2	12	1	2	96	62	4	2	11	2	2
82-2	Japan	ND	97	39	6	2	13	2	2	97	41	7	2	11	3	2
CIA-1	USA	ND	97	30	3	1	12	1	1	98	31	5	1	11	2	1
26P4	USA	6	98	27	6	4	10	2	4	98	27	7	4	8	3	4
BD-3	Bangladesh	NP	97	31	4	2	7	1	2	97	34	4	2	9	2	2
CAF475	China	ND	98	20	3	1	7	2	1	98	25	4	1	7	3	1

ND = not done; NP = non-passaged isolate.

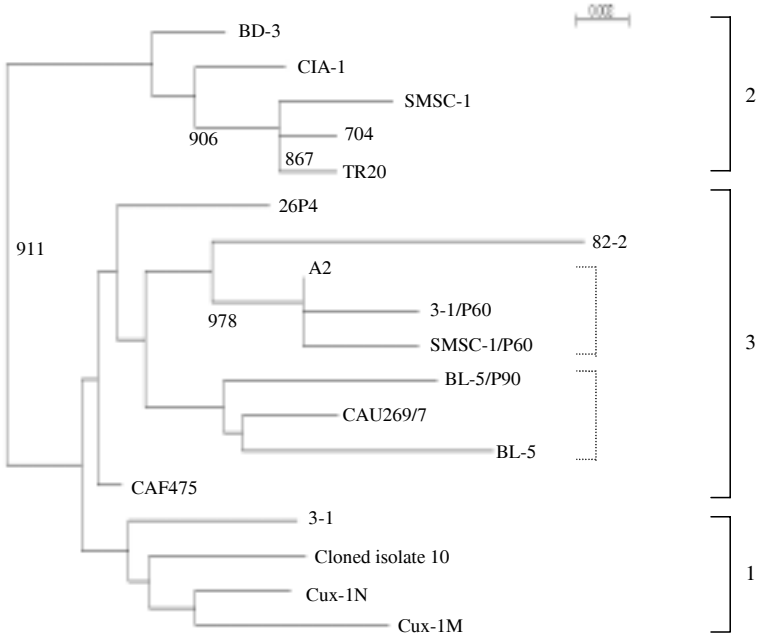

Fig. 1

Phylogenetic tree based on deduced amino acid sequences of VP1 of BL-5, BL-5/P90 and other CAV isolates

Bootstrap probabilities, expressed as percentage of 1,000 resamplings and higher than 800, are given beside the nodes. Three distinct clusters of isolates are designated as 1, 2 and 3. The presence of two subclusters in the cluster 3 is indicated by dotted lines.

in the nucleotide sequence of VP3. Earlier it has been shown that a very highly passaged Cux-1 isolate, the cloned isolate 10, which had undergone 173 passages, displayed only 17 nucleotide substitutions dispersed throughout the whole genome (Meehan *et al.*, 1997). These substitutions resulted in 7, 4 and 3 amino acid changes in VP1, VP2 and VP3, respectively. However, we have shown earlier that the highly passaged isolates SMSC-1/P60 and 3-1/P60 differed from the parental low-passaged isolates SMSC-1 and 3-1 by a high number of nucleotide substitutions (65 and 33, respectively, located throughout the coding region of the genome) (Chowdhury *et al.*, 2003). However, most of the nucleotide substitutions were synonymous mutations resulting in only 13–16 amino acid changes, mainly in the VP1 region.

The isolates BL-5 and BL-5/P90 also showed the highest similarity (98%) with the isolates CAF475 and A2 (Table 1) with 24–32 nucleotide substitutions resulting in 10–12 amino acid changes. Meanwhile, both the isolates showed the lowest similarity (95%) with the isolates 704, TR20 and SMSC-1 with 67–69 nucleotide substitutions resulting in 14–16 amino acid changes located primarily in VP1. The isolates BL-5 and BL-5/P90 showed the highest number of nucleotide substitutions, 82 and 84, respectively, resulting in only 13 and 14 amino acid changes, respectively, as compared to the Australian isolate CAU269/7. However, these substitutions were associated with only 6 amino acid changes in VP1. The calculated non-synonymous (NS) to synonymous (S) mutations ratio in VP1 for the isolates BL-5 and BL-5/P90 compared to other CAV isolates except for CAU269/7 was 0.24–0.81, while the NS/S ratio for BL-5 and BL-5/P90 compared to CAU296/7 was only 0.01. However, both the isolates showed 5 amino acid changes in VP3 compared to CAU269/7 (data not shown). The genetic characteristics of the isolates BL-5 and BL-5P90 with unusually low and high NS mutations in VP1 and VP3, respectively, compared to CAU269/7 deserve further studies.

We have shown earlier that the highly passaged isolates SMSC-1/P60 and 3-1/P60 grouped separately but closely to the cluster of low passaged isolates SMSC-1 and 3-1 (Chowdhury *et al.*, 2003). However, in this study, the highly passaged isolate BL-5/P90 grouped together with the isolates BL-5 and CAU269/7 in the cluster 3 (Fig. 1). The isolates BL-5 and BL-5P90 grouped in the cluster 3 also with the isolates SMSC-1/P60 and 3-1/P60, but in a separate subcluster. It seems that the CAV isolates in the cluster 3 did not group as tightly as the isolates in the clusters 1 and 2 (Fig. 1). Interestingly, the three distinct clusters observed in the phylogenetic tree correlate well with some key amino acids in VP1 (Table 2). E.g., the isolates BL-5 and BL-5/P90 which grouped with other CAV isolates in the cluster 3 shared similar amino acids at positions 75 (Val/Ile), 97 (Met), 139

Table 2. Comparison of amino acid substitutions in BL-5, BL-5/P90 and other CAV isolates

Isolate/ passage	Country of origin	Amino acid change in protein at position																
		VP1												VP2			VP3	
		75	97	139	141	144	157	251	254	287	370	413	447	35	153	187	12	118
BL-5	Malaysia	V	M	K	Q	E	V	R	E	T	G	S	S	S	V	D	Q	C
BL-5/P90	Malaysia	V	M	K	Q	K	V	R	G	T	G	S	S	L	V	D	Q	C
SMSC-1	Malaysia	I	L	Q	Q	Q	V	R	E	T	S	A	S	S	V	D	P	C
3-1	Malaysia	V	M	K	Q	E	V	Q	E	D	S	A	T	S	V	N	P	C
SMSC-1/P60	Malaysia	V	M	K	E	E	M	R	E	S	G	A	T	S	A	D	P	R
3-1/P60	Malaysia	V	M	K	E	E	M	R	E	S	G	A	T	S	A	D	P	R
Cux-1M	Germany	V	M	K	Q	D	V	Q	E	A	S	A	T	S	V	N	P	C
Cux-1N	Germany	V	M	K	Q	D	V	Q	G	A	S	A	T	S	A	D	P	R
Cloned isolate 10	Germany	V	M	K	L	E	V	Q	Q	A	S	A	T	L	A	D	P	R
CAU269/7	Australia	V	M	K	Q	E	V	R	E	T	R	S	S	S	V	D	P	C
704	Australia	I	L	Q	Q	Q	V	R	E	T	S	A	S	S	V	D	P	C
A2	Japan	V	M	K	E	E	M	R	E	S	G	A	T	S	V	D	P	C
TR20	Japan	I	L	Q	Q	Q	V	R	E	T	S	A	S	S	V	D	P	C
82-2	Japan	I	M	K	E	E	V	R	G	S	G	S	T	S	V	D	P	C
CIA-1	USA	I	L	Q	Q	Q	V	R	E	A	S	A	S	S	V	D	P	C
26P4	USA	V	M	K	Q	E	M	R	G	T	S	A	T	S	V	D	P	C
BD-3	Bangladesh	I	L	Q	Q	Q	V	R	E	A	T	A	T	S	V	D	P	C
CAF475	China	V	M	K	Q	E	V	R	E	T	S	A	T	S	V	D	P	C

(Lys) and 144 (Glu/Lys) with the CAV isolates in the cluster 1 (Table 2). However, the CAV isolates grouped in the cluster 2 have distinct amino acids at positions 75 (Ile), 97 (Leu), 139 (Gln) and 144 (Gln).

A comparison of BL-5 and BL-5/P90 showed 9 unique amino acid changes, of which 7 were in VP1 (G92D, A115V, E144K, L202V, Q211H, V224A, and T315P) and 2 were in VP2 (A28G and E175G) (data not shown). The isolate BL-5/P90 showed also the same amino acid changes, E254G in VP1 and S35L in VP2, as those found in Cux-1N, 82-2, 26P4 and Cloned isolate 10 (Table 2). In VP1, both the isolates BL-5 and BL-5/P90 shared three similar amino acids changes (S370G, A413S, and T447S), while in VP3, both the isolates showed just one amino acid change (P12Q). Most of the substitutions were in VP1 with only 1 and 3 substitutions in VP2 and VP3, respectively. The reduced virus pathogenicity as a possible result of these amino acid substitutions remains to be proved since studies characterizing the pathogenicity of the isolate BL-5/P90 for chickens are so far missing. The isolate BL-5/P90 may not show a reduced pathogenicity *in vivo* since it lacks the particular amino acid changes V153A in VP2 and C118R in VP3 as found in other highly passaged isolates, namely SMSC-1/P60, 3-1/P60 and Cloned Isolate 10, known for a reduced pathogenicity (Chowdhury *et al.*, 2003; Meehan *et al.*, 1997).

The overall phylogenetical clustering of different CAV isolates presented in Fig. 1 was very similar to previous one based on the entire sequence of the virus genome (Chowdhury *et al.*, 2003) or just on the sequence of VP1 (Islam *et al.,* 2002), indicating that the relationships of different CAV isolates can be determined on the basis of VP1. The clustering of BL-5 distantly to SMSC-1 and 3-1 suggests that in Malaysia CAV isolates with different genetic characteristic can be found. However, sequence and phylogenetic analyses of CAV should be carefully evaluated since genetic changes associated with low and high passages are not clear. Nevertheless, it seems that regardless of the virus origin, a fairly similar number of amino acid changes were realized in highly passaged isolates indicating a role of selection pressure in adapting the virus to grow in cultured cells.

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