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MOLECULAR CHARACTERIZATION OF THE σC-ENCODING GENE FROM NOVEL DUCK REOVIRUS

T. Yun[1], H. P. Chen[2], J. G. Hua[1], B. Yu[1], L. Chen[1], Z. Ni[1], W. C. Ye[1] and C. Zhang [1*]

[1]Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China
[2]College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai•an 271018, China
[*]Corresponding author Email Address: zhangcun@aliyun.com

ABSTRACT

We cloned and sequenced the σC-encoding gene of a novel duck reovirus (N-DRV) isolate ZJ00M. The σC gene is 966 bp long and is encoded by the third ORF of the S1 segment, a small protein of 321 amino acid residues (approximately 34.1 kDa). Expressed σC fusion protein in E. coli showed good antigenicity and immunogenicity in a western blotting assay and indirect immunofluorescence assays (IFA). The comparative sequence analyses indicated that N-DRV σC shared high identity levels with ARV species (classical MDRV and ARV) and a lower degree of identity with other orthoreovirus counterparts, but the sequence identities were no more than 45 %. As observed in classical MDRV and ARV σC protein sequences, some sequence motifs, conserved sequence motifs and/or functional domains were identified in the sequence of N-DRV σC. The phylogenetic analysis of σC/σ1 sequence revealed that N-DRV was different from other orthoreovirus species groups but remained classified into the avian orthoreoviruses along with other ARVs, suggesting that N-DRV and classical MDRVs constitute a separate geno group relative to ARV. These results are important for future studies examining the structure, function and immunogenicity of N-DRV σC.

Keywords: Novel duck reovirus, σC-encoding gene, Expression, Phylogenetic analysis, Genotype

INTRODUCTION

The avian reoviruses (ARVs) belong to the *Orthoreovirus* genus of *Reoviridae* family, and are ubiquitous in nature. Avian reovirions are non-enveloped, which makes them resistant to adverse conditions, and contain 10 segmented double-stranded RNA (dsRNA) surrounded by a double layer concentric capsid shell with a diameter of 70 to 80 nm. The genome segments can be divided into 3 size classes corresponding to electrophoretic mobilities: large segments (L1, L2, and L3), medium segments (M1, M2, and M3) and small segments (S1, S2, S3, and S4) (Benavente *et al*. 2007; Gouvea & Schnitzer, 1982; Nick *et al*. 1975; Spandidos and Graham, 1976). All avian reovirus genomes encode at least 12 primary proteins, including 8 structural proteins and 4 nonstructural proteins.

Classical Muscovy duck reovirus (MDRV) was initially described as an etiological agent of disease in South Africa in 1950 (Kaschula, 1950) and isolated in France in 1972 (Gaudry *et al*. 1972). Classical MDRV mainly affects ducklings and goslings between 2 and 4 weeks of age. MDRV can cause high morbidity and up to 50% mortality. Recovered Muscovy ducks have markedly stunted growth. The disease is characterized by growth retardation, diarrhea, general weakness, pericarditis, especially liver and spleen covered with small white necrotic foci, for which the disease was designated Muscovy duck white spot disease or Muscovy duck flower liver disease in China (Gaudry *et al*. 1972; Hu *et al*.2004; Malkinson *et al*. 1981; Marius-Jestin *et al*. 1988; Yun *et al*. 2014).

Classical MDRV has many biological properties with ARV, such as its fusogenic nature and the inability to agglutinate red blood cells (Malkinson *et al*. 1981). They also differ from ARV in many aspects, including host species, pathogenic properties (Marius-Jestin *et al*.1988), antigenicity (Heffels-Redmann *et al.*, 1992), electropherotypes, genomic coding assignments (Kuntz-Simom *et al*. 2002) and protein profiles (Heffels-Redmann *et al*. 1992) etc. The minor outer capsid protein of classical MDRV σC is encoded by the S4 segment, which contain two overlapping ORFs (encoded p10 and σC, respectively) (Kuntz-Simom *et al*. 2002), and the protein in ARV is encoded by the S1 segment, which is a tricistronic gene, which expresses p10, p17 and σC.

Since 2000, a new type duck reovirus (N-DRV) disease emerged in the Southeast provinces (the major duck-producing regions) of China, including Zhejiang, Fujian and Guangdong. It was reported that the disease could affect different breeding ducks and geese, and the disease was characterized mainly by hemorrhage and necrotic lesions in the liver and spleen (Chen *et al*. 2012; Huang *et al*.2009; Wang *et al*. 2002; Yun *et al*. 2012, 2014). The sequences of the 10 genome segments of N-DRV have recently been completely determined, and the segment sequences analyses showed that the S1 segment of N-DRV contains three sequential overlapping ORFs, encoding p10, p18 and σC. The structure of N-DRV S1

segment was similar to ARV, which is a tricistronic gene. For classical MDRV, S4 segment is polycistronic gene, which encode the p10 and σC proteins, and is the smallest genome segment among s-class genome segments, and there is no encoding for p18 (Ma *et al*. 2012; Wang *et al*. 2012; Yun *et al*. 2012, 2014). The pathogenicity of N-DRV differs from that of classical MDRV and ARV, and cross-neutralization tests demonstrated that N-DRV does not cross-react with classical MDRV and ARV (Chen *et al*. 2011).

The σC was encoded by the third ORF of the S1 segment in the S-class segments of N-DRV (Ma *et al*. 2012; Wang *et al*. 2012; Yun *et al*. 2012, 2014). The predicted protein (34 kDa) was larger than classical σC (29.5 kDa). The classical MDRV σC plays important role in attachment of virus to the target cells (Martinez-Costas *et al.*, 1997), the production of reovirus-specific neutralizing antibodies (Martínez-Costas *et al*.1997; Shapouri *et al*. 1996; Wickramasinghe *et al*. 1993) and apoptosis (Shih *et al*. 2004). Currently, there are only scarce data concerning the structure and function of N-DRV σC. In this study, we determined, analyzed and expressed the σC-encoding gene from N-DRV isolate in 2000, explored the genetic relationship among classical DRV, ARV and other orthoreovirus genus numbers, and studied antigenicity of the *E. coli*-expressed recombinant N-DRV σC.

MATERIALS AND METHODS

Virus and purification: The N-DRV ZJ00M strain was isolated from the liver sample of a dead Muscovy duck with hemorrhagic-necrotic lesions in 2000 in the Zhejiang Province, as described previously (Yun *et al*. 2012, 2014). Briefly, the homogenized liver suspension was inoculated into the chorioallantoic cavity of 10-day-old Muscovy duck embryos, and then embryos were incubated at 37 °C for 5 days and were monitored daily. For cell isolation, DF-1 cells were inoculated with the collected allantoic fluid, and the cells were examined every day until an observed strong cytopathic effect (CPE). The cell cultures were frozen and thawed 3 times, the cellular debris was removed by low centrifugation at 3000rpm, and the supernatants were stored in aliquots at -80 °Cfor later use.

The virus was purified as previously described (Yun *et al.*, 2012, 2014). The freeze-thawed virus suspension was centrifuged at 4 °C for 30 min at 10,000×gto remove the cell fragment. The supernatants were then precipitated with 50 % saturated ammonium sulfate overnight at 4°C. The precipitates were collected by centrifugation at 10,000×g for 20 min 4 °C and suspended in the TNE buffer (0.02 M Tris (pH 7.0), 0.001 M EDTA, and 0.15 M NaCl). The virus supernatant was then ultracentrifuged on a 40 % sucrose cushion (w/v in phosphate-buffered saline, PBS) for 3 hr at 130,000 ×g at 4 °C in a Beckman SW70 rotor （Beckman Coulter, Fullerton, CA, U.S.A.）. The pellet virions were re-suspended in 50-100 µL of cold DEPC H_2O and stored at −80 °C until use.

Primers: A pair of specific primers for the σC-encoding gene was designed using Oligo 6.24 (Molecular Biology Insights, Inc.) based on the conserved nucleotide sequence from previously reported novel duck reoviruses (N-DRV). The forward primer was N-DRV-SigC F 5' -ACA***CCATGG***ATCGCAACGAGGTGATA CGCCTG-3' (*Nco* I restriction site is underlined), and the reverse primer was N-DRV-SigC R 5'-ATA ***CTCGAG*** GCCCGTGGCGACGGTGAAGCGTAA-3' (*Xho* I restriction site is underlined)

Nucleic acid extraction: The viral dsRNA was extracted using the TRI-Reagent (TaKaRa Biotechnology (Dalian) Co. Ltd) according to the manufacturer's instructions. The extracted RNA was kept at −80 °C.

Cloning and sequencing of the σC-encoding gene: The σC-encoding gene (nucleotides 571 to 1533) was amplified by a Prime Script one step RT-PCR kit (TaKaRa Biotechnology (Dalian) Co. Ltd). Purified double-stranded RNA (2 µl) was mixed with the primers N-DRV-SigC F and N-DRV-SigC R, and the mixtures were denatured at 95 ℃ for 5 min, chilled on ice for 2 min, and used as a template to generate cDNA. One-step RT-PCR was performed in accordance with the PrimeScript one step RT-PCR kit protocol. In the RT-PCR, chilled RNA and primer mixture (6 µl) were used in a 50-µl final reaction volume, with final concentrations of 1×1 One-step buffer, 2 µl of Prime Script 1 Step Enzyme Mix, RNase free H_2O added to a final volume of 50 µl. The One-step RT-PCR was carried out at 50 °C for 30 min, then at 95°C for 30 min, followed by 30 cycles of 94 °C for 30 s, 55°C for 30 s and 72 °C for 1 min. The amplified products were separated on a 1 % agarose gel, and a 963 bp size fragment of interest was obtained, excised and purified with a gel extraction kit (TaKaRa Biotechnology (Dalian) Co. Ltd). The σC-encoding gene was cloned into the plasmid pMD18-T vector (TaKaRa Biotechnology (Dalian) Co. Ltd). The positive clone identified by PCR was further confirmed by sequencing.

σC-encoding gene expression and protein purification: To generate a plasmid that expressed the His-tagged fusion protein, the pMD-σC plasmid and the pET-28 expression vector (Novagen, USA) were digested with *Nco* I and *Xho* I restriction enzymes. The double digested σC fragment was cloned into digested pET-28a to yield the recombinant plasmid (designated pET28-σC). The plasmid was transformed into E. coli strain BL21 (DE3). The positive strain was grown in LB medium (50 µg/ml kanamycin) and cultured at 37 ℃ overnight. After induction with a 1 mM final concentration isopropyl-β-D-thioga-lactopyranoside (IPTG) for 5 h, the bacterial cells

were collected by centrifugation, and the cell pellets were dissolved in a 1/10 volume of binding buffer with denaturing buffer (0.01 mM Tris-cl, 0.1 M sodium phosphate, 8 M urea, pH8.0; 200 µg/mL lysozyme) followed by freeze-thawing cycles and sonication. After centrifugation (fractionation) at 13,000 rpm for 15 min at 4 °C, the supernatant of the crude extract containing the inclusion bodies was further purified using a Ni-NTA kit (Novagen, USA). The purification was performed according to the manufacturer's protocol. The protein concentration was quantified using the Quawell protein assay (Quawell Technology, CA, USA), and the purity was monitored by SDS-PAGE.

Ethics statement: The duck studies were approved by the Animal Care and Use Committee of Institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences, and were carried out in accordance with the regulations and guidelines of the Zhejiang Academy of Agricultural Sciences.

Polyclonal sera: The anti-duck reovirus polyclonal serum was obtained from sacrificed SPF Muscovy ducks experimentally infected with the N-DRV ZJ00M strain. Anti-σC polyclonal serum was obtained from sacrificed New Zealand white rabbits (Lab Animal Center, Zhejiang Academy of Agricultural Sciences) immunized with the σC protein excised from a SDS-PAGE gel. The N-DRV-negative sera were collected from SPF Muscovy ducks. The anti-classical MDRV, ARV, H5N1 AIV, duck herpsevirus and duck parvovirus sera were provided by the institute of Animal Husbandry and Veterinary Sciences, Zhejiang Academy of Agricultural Sciences.

Characterization of the expressed σC protein: The purified σC protein extracts from the induced *E. coli* strain BL21(DE3) were resolved by 10% SDS-PAGE and electrotransferred to PVDF membranes (BioRad, Hercules, CA, USA) using semi-dry electroblotting. The membranes were blocked in PBST containing 5% dried skimmed milk and incubated with duck hyperimmune sera against N-DRV (diluted 1:1000) in PBST overnight at 4°C. After several washes with PBST buffer, the membranes were incubated with the mouse anti-duck immunoglobulin MoAb conjugated with horseradish peroxidase (1:5000 dilutions, kept in our lab) for 1 h at 37 °C. After PBST washing to remove the unbound antibodies, the membrane was incubated with the substrate 3, 3'-diaminobenzidine tetrahydrochloride (20 ml 0.1 M pH 7.4 Tris-HCL, 20 mg DAB, and 0.005 % H_2O_2).

For the indirect immunofluorescence assay (IFA), DF-1 cells were infected with the N-DRV ZJ00M strain, fixed 48 h after infection in 3.7 % paraformaldehyde in PBS (pH 7.5) at room temperature for 30 min and permeabilized by incubation in -20 °C methanol for 30 min. The fixed cells were washed 3 times with 0.1 % NP40-PBS and incubated with a rabbit anti-N-DRV-σC polyclonal antibody for 1 h at 37°C, respectively. After incubation with the primary antibody, the cells were washed 3 times with 0.1 % NP40-PBS and incubated for 1 h at 37 °C with a 1:1000 dilution of a FITC-labeled goat anti-rabbit IgG (Zhongshanjinqiao Biotechnology Co., Ltd.), respectively. The cells were observed through a fluorescence microscope (Nikon 80i, Tokyo, Japan).

Analysis of the nucleotide and deduced amino acid sequences: The nucleotide and deduced amino acid sequences of the N-DRV σC-encoding gene were analyzed using the DNASTAR software package (Version 7.1.0; DNASTAR, Inc.). Comparisons of the sequences with the sequences available in databases were performed using the NCBI blast program (http:// www. ncbi.nlm.nih.gov /BLAST/). Multiple sequence alignments were performed using Clustal-W. Searches for biologically relevant sites were performed by scanning the sequences against the prosite database. Phylogenetic trees were constructed using the Neighbor-Joining and tested with 1000 bootstrap replicates in MEGA version 5 (Tamura *et al*., 2011). The alignments and comparisons of the available sequences of σC/σ1 from GenBank (including ARV, DRV, NBV, RRV, Pulau and MRV1-3) were performed by MEGA 5. The accession numbers of the strains sequenced in this study are shown in Table 1.

RESULTS

Characterization of DRV σC-encoding gene segment and paired identities to other orthoreoviruses: For the σC protein, sequence comparisons showed that the deduced amino acid sequence of the N-DRV σC protein shared low identities with the σC homolog of ARVs (29-48%), including ARV, GRV and MDRV. The analysis of the structural and functional motifs showed that the N-DRV σC protein possessed several phosphorylation and N-glycosylation sites, but no myristoylation sites (data not shown). The N-DRV σC did not contain a leucine zipper motif, which has been reported in the ARV, classical MDRV/GRV and MRV counterparts. It was predicted that the N-DRV σC protein had a slightly acidic isoelectric point of 5.995, with a mainly hydrophilic portion located in the middle part and a mainly hydrophobic portion located within the extreme N-terminus. The predicted secondary structure of the N-DRV σC has the following features: 27.41% of the residues are in the form of α-helices, 17.45% are in the form of extended strands and 55.14% are in random coils. Using the MultiCoil program, Examination of the amino acid sequence of the N-DRV σC showed that there is a heptapeptide repeat pattern (a–b–c–d–e–f–g),between amino acids 21 – 146 (Fig 1), and the mainly hydrophobic amino acids are located in the first and

fourth positions (a and d) (Table 2). Approximately 56.8% of the α-helices are in the N-terminal half of the molecule and are not uniformly distributed. The amino acid alignments showed that there are only forty conserved amino acid residues among ARV, classical MDRV/GRV and N-DRV/N-GRV, including three conserved proline (P) residues at positions 169,183 and 271, and a conserved cysteine residue (Cys186). The conserved residues were almost located in the C-proximal domain (Fig. 1).

The nucleotide and deduced amino acid sequences of N-DRV ZJ00M σC were aligned with those of the homologous protein from other members of the *orthoreovirus* genus. The aligned sequences were further confirmed by pairwise comparisons, and the percentage of sequence identities were determined (Table 3). The results showed that N-DRV ZJ00M had a higher similarity (nt, 36.7-37.2%; AA, 40.5-41.3%) with the avian orthoreoviruses (ARV and classical MDRV) than did NBV, Pulau, RRV and MRV of orthoreovirus genus. The sequence identities were not greater than 45%.

Phylogenetic analysis: To analyze further the evolutionary relationship between N-DRV and other *Orthoreovirus* genus members, a phylogenetic analysis was performed. Amino acid sequences were available for the majority of the orthoreovirus species groups. Phylogenetic trees constructed using the Neighbor-Joining method revealed that, based on the nucleotide and putative amino acid sequences of the σC/σ1 of representative viruses from the *Orthoreovirus* genus, N-DRV ZJ00M was classified into the previously defined *Orthoreovirus* species group Ⅱ along with the other ARVs, and the protein was grouped further into the classical MDRV and GRV genogroup (Fig. 2).

Table 1.Accession numbers of orthoreovirus genus used in this study

Orthoreovirus Species groups	Virus	Strain	Origin	Isolate year	Host	GenBank accession number of σC/σ1
Ⅱ	ARV	S1133	USA	1973	Chicken	AF330703
		176	USA	N	Chicken	AF218358
		138	Canada	N	Chicken	AF218359
	Classical MDRV	ZJ2000M	China	2000	Muscovy duck	KF306091
		815-12	China	2010	Muscovy duck	KC508656
		S14	China	1997	Muscovy duck	DQ066925
		89026	France	1989	Muscovy duck	AJ310525
		89330	France	1989	Muscovy duck	AJ251834
		MW9710	China	1997	Muscovy duck	AY580159
		ZJ99	China	1999	Muscovy duck	AY619690
	Classical GRV	D14/99	Hungary	1999	Goose	AJ717735
		D20/99	Hungary	1999	Goose	AJ717737
		D34/99	Huagary	1999	Goose	AJ717738
	N-GRV	03G	China	2003	Goose	JX145334
	N-MDRV	ZJ00M	China	2000	Muscovy duck	KF154114
		091	China	2009	Pekin duck	JX478256
		J18	China	2008	Muscovy duck	JX478266
		TH11	China	2011	Pekin duck	JX826587
		NP03	China	2009	Muscovy duck	KC312699
Ⅳ	BRoV	N	Australia	N	little red flying fox	NE
	BRV	N	USA	N	Papio cynocephalus	NE
Ⅲ	NBV	N	Australia	N	Pteropus cynocephalus poliocephalus	AF218360
	Pulau	N	Malaysia	N	Pteropus hypomelanus	AY357730
Ⅴ	RRV	N	Canada	N	Python	AY238887
Ⅰ	MRV1	Lang	N	N	Homo sapiens	M14779
	MRV2	MRV2Tou05	France	N	Homo sapiens	GU196315
	MRV3	Dearing	N	N	Homo sapiens	NC004277

N: unknown. NE: no equivalent sequence.

Expression and antigenicity of N-DRV ZJ00M σC protein: To obtain over-expressed recombinant protein σC, we constructed a prokaryotic expression plasmid (pET28-σC). The SDS-PAGE analysis revealed that the expressed recombinant fusion protein was approximately 36 kDa, which was consistent with the expected size of the fusion protein containing the N-terminal tag, and the protein was highly expressed after induction by 1 mmol/L IPTG at 37 °C for 5 h. (Fig. 3A). The analysis of the supernatant and pellet fraction by SDS-PAGE suggested that the expressed protein was assembled into inclusion bodies. The expressed fusion protein was analyzed by western blot analysis with N-DRV polyclonal antiserum, and the expressed anti-His-tagged σC fusion protein with an approximate molecular weight of 36 kDa could react specifically with the polyclonal serum (Fig. 3B). No specific N-DRV protein was detected in the lysates derived from pET-28a empty vector transformed BL-21(DE3) cells (Fig. 3B). These results show that the expressed σC fusion protein was an antigenic protein.

The indirect immunofluorescence assays (IFA) demonstrated that there was strong fluorescence observed in the cytoplasm of the N-DRV infected cells and not in the nucleus (Fig. 4A). No specific fluorescence was observed in the mock-infected cells (Fig. 4B). These results indicate that anti-His-tagged σC sera have good reactivity and specificity against N-DRV.

Table 2. Heptapeptide repeat pattern for the amino acid sequence of σC protein of N-DRV

a	b	c	D	e	f	g	Amino acid number
V	D	H	L	T	T	Q	21-27
I	K	S	L	Q	S	A	28-34
V	D	S	L	K	E	S	35-41
Q	V	V	V	L	R	R	42-48
L	T	T	I	T	S	T	49-55
V	A	D	L	Q	S	T	56-62
T	E	L	L	T	S	Q	63-69
V	A	G	L	S	S	R	70-76
V	A	S	V	T	D	E	77-83
V	V	R	V	D	S	V	84-90
I	G	S	T	I	T	N	91-97
L	D	N	V	R	S	E	98-104
L	S	S	L	S	S	Q	105-111
V	S	S	Q	T	S	T	112-118
L	T	N	L	T	S	T	119-125
V	S	S	Q	S	L	A	126-132
I	S	D	L	Q	R	R	133-139
V	T	V	L	E	R	S	140-146
17/18	2/18	3/18	15/18	2/18	1/18	1/18	Number of hydrophobic amino acids
94	11	17	83	11	6	6	Percentage of hydrophobic amino acids

Hydrophobic residues are underlined

Table 3 Percentage identities of N-DRV σC and homologous encoded proteins of ARV, classical MDRV and other orthoreovirus

	ZJ00M	89330	S14	ZJ2000M	S1133	138	176	NBV	Pulau	RRV	MRV1	MRV2	MRV3
ZJ00M		40.5	41.3	40.9	26.5	27.4	26.8	21.2	17.8	17.4	16.5	15.3	13.7
89330	**36.7**		93.3	93.3	22.7	21.2	22.7	16.0	16.7	17.8	14.1	16.4	14.5
S14	**37.2**	**92.7**		97.8	21.9	21.2	21.9	16.0	17.5	16.4	14.5	17.1	14.9
ZJ2000M	**36.9**	**93.1**	**98.6**		21.6	21.6	21.6	16.4	17.5	16.0	15.6	16.0	14.5
S1133	**28.7**	**24.2**	**24.2**	**23.8**		84.0	98.8	23.2	22.4	16.6	15.3	15.6	15.3
138	**28.2**	**23.6**	**24.1**	**25.3**	**78.6**		84.7	22.6	22.7	16.6	14.4	15.0	15.3
176	**29.1**	**24.3**	**24.4**	**24.0**	**99.2**	**79.3**		23.5	22.1	17.2	15.3	14.4	15.3
NBV	**23.0**	**24.6**	**24.4**	**22.8**	**27.8**	**28.0**	**27.5**		38.4	13.9	16.7	14.9	16.1
Pulau	**22.5**	**22.0**	**23.1**	**23.1**	**27.3**	**28.2**	**27.4**	**38.9**		15.0	13.5	15.0	14.4
RRV	**24.4**	**21.1**	**20.7**	**21.1**	**23.2**	**21.8**	**23.5**	**21.9**	**20.8**		14.0	15.8	13.8
MRV1	**20.5**	**21.6**	**21.5**	**22.3**	**22.5**	**22.3**	**22.7**	**21.4**	**21.6**	**20.8**		46.9	21.3
MRV2	**20.6**	**20.7**	**22.2**	**21.6**	**22.5**	**21.0**	**21.7**	**20.9**	**21.5**	**22.4**	**47.3**		22.0
MRV3	**22.**	**20.5**	**21.6**	**21.9**	**21.4**	**23.2**	**21.0**	**20.6**	**20.4**	**22.3**	**24.8**	**29.2**	

Fig.1. N-DRV σC protein deduced amino acid sequences (strains ZJ00M) and their alignments with homologous minor outer capsid proteins (classical MDRV and ARV). Amino acid positions for each individual sequence are numbered on the right. Identical amino acids in all the aligned sequences are indicated by a triangle (▲).Boxed with a bold line indicates the leucine zipper patterns. The first and last residues of the major heptad repeat patterns are underline. Open circles over the aligned sequences indicate the location of conserved proline and cysteine residues and filled circles indicate the location of conserved hydrophobic residues.

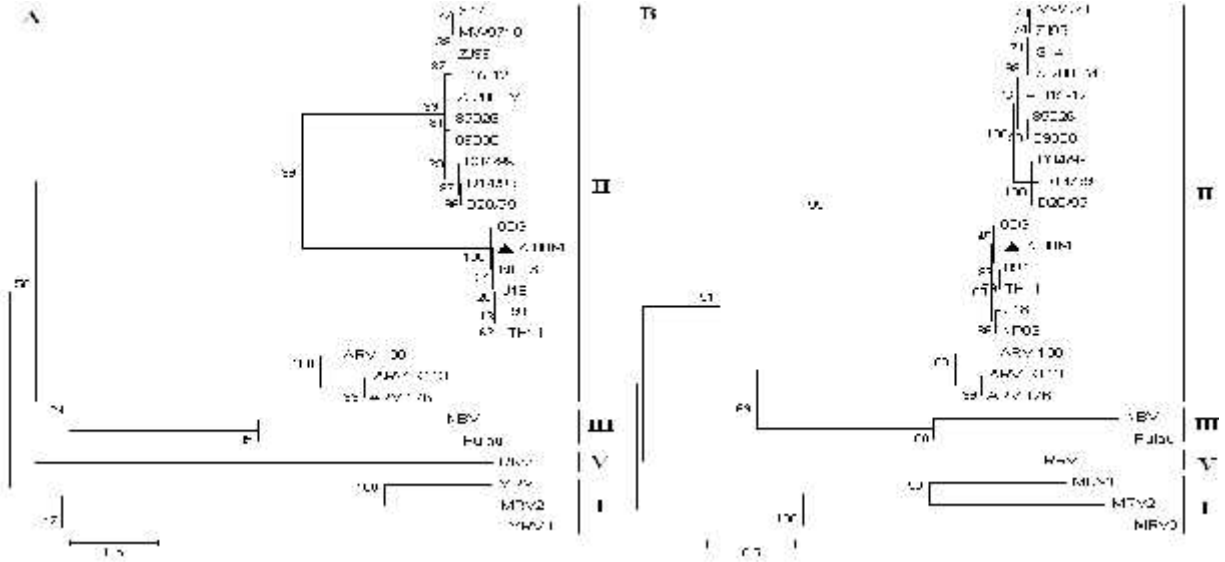

Fig.2. Phylogenetic trees built based on nucleotide (A) and amino acid (B) sequences of σC of members of Orthoreovirus, using the Neighbor-Joining method in the mega 5 program. Bootstrap values of 1000 replications are shown at the notes. The Orthoreovirus genus groups Ⅰ, Ⅱ, Ⅲ and Ⅴ, defined according to the Eighth Report of the ICTV, are indicated on the right-hand side of each cladogram. (▲) Strain determined in this study.

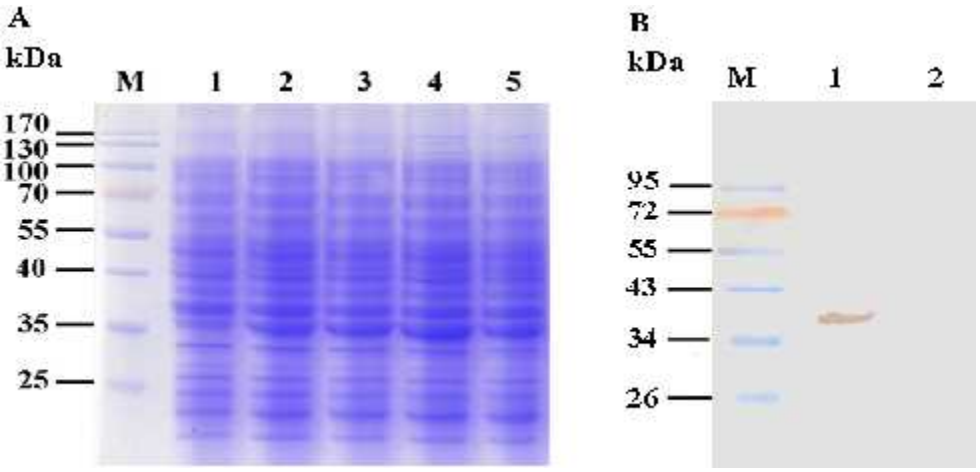

Fig.3. The expressed N-DRV σC protein analysis by SDS–PAGE and Western blot. (A) Whole cells were lysed and the pelleted materials where used. lane 1 (before IPTG-induction), lanes 2-5 (3, 4, 5 and 6 h after IPTG-induction, respectively). All samples were boiled in Laemmli sample buffer were loaded on a 10% SDS–PAGE gel, and then proteins were either stained with Coomassie blue (A) or transferred to a PVDF membrane, probed with a reovirus-specific muscovy duck antiserum, and detected with the horseradish peroxidase system (B) (lane 1, pET28-σC, lane 2, pET28a vector). Molecular weight markers are shown in lane M.

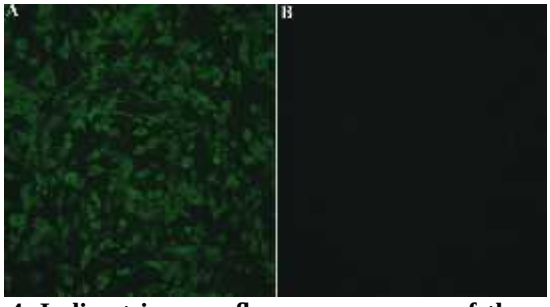

Fig.4. Indirect immunofluorescence assay of the anti-His-tagged σC serum in DF-1 cells infected by N-DRV ZJ00M. (A) N-DRV infected DF-1 cells probed with the anti-N-DRV-σC polyclonal antibody; (B) Mock infected DF-1 cells probed with the anti- N-DRV-σC polyclonal antibody.

DISUSSION

The N-DRV is distinct from the classical MDRV in the S-class genome segment that encodes the σC. The σC is encoded by the S1 genome segment in the N-DRV and by the S4 genome segment in the MDRV. The organization of the polycistronic S1 genome segment in the N-DRV is similar to that in the ARV. σC/σ1 is a cell attachment protein, and the protein determines tissue-tropism, induces neutralization antibodies, carries serotype specificity, and can agglutinate red blood cells in the case of MRV (Mertens, 2004). Because of selective pressures, the σC-encoding gene displays the highest level of sequence divergence and rapid evolution, and it is highly diverse even within an orthoreo virus species (Attoui *et al*. 2001; Kuntz-Simom *et al*. 2002; Shapouri *et al*. 1995), but its multimer structure and globular-like head figure is conserved in all the orthoreo viruses (Bαnyai *et al*. 2005).

The N-DRV σC had a heptapeptide repeat (a–b–c–d–e–f–g) based on the amino acid sequences and secondary structure prediction analysis. The presence of hydrophobic amino acids at positions (a and d) had been associated with a specific configuration, in which α-helical regions of the protein formed coiled-coil super helices and the α-helicals are wound around each other (Lupas, 1996, 1997). It was predicted that the structural features may be responsible for oligomerzaiton and incorporation of σB into virions (Shapouri *et al*. 1995).The N-DRV σC did not contained the leucine zipper motif that was located in the N-terminal region, which would be involved in the oligomerization of the protein in ARV, classical MDRV (GRV) and MRV counterparts (Belli & Samuel, 1993; Kuntz-Simom *et al*. 2002; Vakharia *et al*. 1996). The N-DRV σC possessed several N-glycosylation and phosphorylation sites but no myristoylation sites. These motifs and sites are not identical with classical MDRV and ARV. The alignment of the N-DRV, classical MDRV and ARV σC proteins revealed that the conserved residues were located in the C- terminal half of the protein, including three proline residues and a cysteine residue that might play a vital role in protein structure, bordering well-defined morphologic regions (Kuntz-Simom *et al*. 2002). This finding suggested that the N-DRV σC protein shared functional and structural similarities with classical MDRV and ARV homologous proteins and their cell binding domain(s)/ sites located on the tail domain. A divergence analysis of

the σC-encoding gene of N-DRV, classical MDRV and ARV would be allow for a better correlation between the serologic and genetic classifications.

Reoviruses have been classified based on their morphological and genetic features. Comparisons of the nucleotide and amino acid sequence identity of homologous genome segments and encoded proteins is an important criterion for virus classification (Wang *et al*.2013; Yun *et al*. 2013). Viruses that share more than 75% nt (between homologous genome segments) and 85% aa (between conserved core proteins) identities can be classified into the same species groups, but if the nucleotide and amino acid identities are less than 60% and 65%, the viruses should be considered in different species groups (Chappell *et al*. 2005). The amino acid identity between the outer capsid proteins must be more than 55% for viruses within a species and less than 35% to be considered a different species (Chappell *et al*. 2005). According to the classification criteria, the amino acid identity of the N-DRV σC protein was only 15.9-42.0% with ARV, classical MDRV and other members of the *Orthoreovirus* genus, and N-DRV ZJ00M should be considered as a different species than ARV. The phylogenetic analysis based on the nucleotide and amino acid sequences of σC of representative viruses from the *Orthoreovirus* genus showed that the N-DRV ZJ00M was still classified into the previously defined *Orthoreovirus* species group Ⅱ along with the other ARVs (including DRV and GRV), and grouped further into classical MDRV and GRV genogroups.

In this study, we successfully cloned the σC gene, characterized its structure (Tables 1 and 2), and expressed the N-DRV σC protein in the prokaryotic cells for the first time (Fig 3). The recombinant fusion protein over-expressed, appeared in the formation of inclusion body, and reacted specifically with the N-DRV antiserum. The anti-σC sera have good reactivity and specificity against N-DRV infected DF-1 cells. The results suggested that the expressed σC protein possessed the antigenicity and could be used for vaccinology (Kuntz-Simon *et al*. 2002). The heterogenetic expression of the σC gene and its optimization in the bacteria could facilitate future studies investigating the biological synthesis in vitro, molecular structure and function, immunogenicity and subunit vaccine of the N-DRV σC protein.

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