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The interferon antagonistic activities of the V proteins of NDV correlated with their virulence

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Abstract

Protein V of Newcastle disease virus (NDV) serves as interferon (IFN) antagonist, and NDV stains with different pathogenicity show different abilities in inhibition IFN expression. To further reveal the relationship between viral virulence and their IFN-antagonistic activity derived from protein V, six NDV strains with three different pathotypes were used in this study and their V gene were cloned into eukaryotic expression vector. The V gene derived from different NDV strains were expressed in same level in cells after transfection according to the results from Western blotting. And these proteins showed different interferon-antagonistic activities based on interferon expression using Luciferase Reporter Assay and ELISA. The expression of IFN and viral virulence index, mean death time, have a good linear relationship indicating a good correlation between viral virulence and IFN antagonism of their V Protein.

Keywords Newcastle disease virus (NDV) · Non-structural protein · Protein V · Antagonistic interferon · Virulence

Abbreviations

IFN	Interferon
NDV	Newcastle disease virus
ND	Newcastle disease
SV	Sendai virus
SV5	Simian virus 5
CDV	Canine distemper virus
RPV	Rinderpest virus
RSV	Respiratory syncytial virus
NP	Nucleocapsid protein
P	Phosphoprotein
M	Matrix protein
F	Fusion protein
HN	Hemagglutinin–neuraminidase protein
L	RNA-dependent RNA polymerase
CTD	C-terminal domain
R	Arginine
E	Glutamic acid
I	Isoleucine
P	Proline
A	Alanine
IFN	Interferon
STAT1	Signal transducer and activator of transcription
MDA-5	Melanoma differentiation-associated protein 5

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Main text

Virulent Newcastle disease virus (NDV), or Avian Paramyxovirus Serotype 1, is the causative agent of one most important avian disease, Newcastle disease (ND), which causes substantial economic losses in the poultry industry worldwide. NDV is a member of genus *Avulavirus* in the subfamily *Paramyxovirinae* within the *Paramyxoviridae* family [1]. Several other members in this family [1], like Sendai virus (SV), simian virus 5 (SV5), canine distemper virus (CDV), rinderpest virus (RPV), and Hendra and Nipah viruses, are also well known for the serious diseases caused by them [11].

Paramyxoviruses have non-segmented negative polarity single-stranded RNA genome [7]. The NDV genome was constituted by 15,186 nucleotides which codes six genes with an order of 3′-NP-P-M-F-HN-L-5′. Six major structural

proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein(HN), and one large RNA-dependent RNA polymerase (L), were expressed in NDV infected cells [7]. Two additional proteins (designated as V and W proteins) were also expressed by an RNA editing mechanism during the transcription of P gene [8].

The V protein of NDV is composed of 239 amino acids with a molecular weight of 36 kDa. At N-terminal, V protein shared 135 amino acids with P, while V protein has a different C-terminal domain (CTD). The CTD of NDV V protein, like other paramyxovirus, is highly conserved and forms a unique zinc finger fold by an invariant histidine (H) and six cysteine (C) residues [10]. NDV V protein was identified as an IFN antagonist and also related to the virulence of NDV [1]. CTD region determines the IFN antagonist activity of V protein. Nevertheless, the functional domain in CTD is conserved and both virulent and non-virulent NDV shared the conserved zinc finger structure in CTD, as well as other paramyxovirus V proteins. According to these findings, it would be interesting to find out whether the V proteins derived from different pathotype NDV strains have similar IFN-antagonistic activities. One previous report using one mesogenic (MG) NDV and one lentogenic (LG) NDV strain indicated that the interferon-antagonist activities of V proteins are related to the virulence of NDV strains [6]. However, too less and no velogenic (VG) NDV strains were used in their studies.

Here, IFN antagonistic activities of the V proteins from three VG (Shaanxi06 [2], sd-08 and F48E9 [9]), two MG (Shaanxi10 [2], Gfw-10), and one LG NDV (HX01 [3]) isolates were compared using luciferase assay. The related information of these V proteins are listed in Table 1, and a sequence alignment based on the amino acids sequences of these V proteins is shown in Fig. 1. Chicken IFN-β promoter sequence (GenBank: Y14969) was inserted into a firefly luciferase report gene vector (vector pGL3-basic) to get a chicken interferon reporter system (pChIFN-β-luc). Vector, pRL-TK, harboring Renilla Luciferase gene was served as the control. By artificially inserting one G in the editing site of P gene using fusion PCR, six V protein coding genes were cloned into the eukaryotic expression vectors, pCMV-3HA. And the protein expressions were detected by western blotting. Briefly, vectors, pCMV-3HA-V, were transfected into chicken fibroblast cell line DF1 cells at 80% confluence in triplicates. After 24 h, the cells were stimulated with 2 μg/mL poly(I:C) by transfection. And then, the cells were collected at 0, 2, 4, 8, 16, 24, and 48 h post-poly(I:C) treatment. And western blotting was conducted to detect the expressions of V by detecting 3HA tag fused to the N terminal of V proteins. The V proteins from different NDV strains show similar expression level at each time points (Fig. 2A). The expression amount of each V protein increased gradually over the time after treatment with poly(I:C) (Fig. 2a). That similar amounts of protein were loaded into each lane was confirmed by comparison the bands of β-actin.

In order to verify the reporter system, DF1 cells at 80% confluence were transfected with pChIFN-β-luc (250 ng) or control vectors (pGL3-basic, 250 ng), along with pRL-TK (10 ng) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. 24 h later, the cells in each well were re-transfected with 2 μg/mL poly(I:C). And after another 24 h, the cells were harvested for detection of luciferase activity using a luciferase detection kit (Beyotime Biotechnology, China). Each assay was conducted with three replicates. The results were presented as the mean ± SD. As shown in Fig. 2b, with the stimulation of poly(I:C), luciferase activities of the cells transfected with pChIFN-β-luc were significantly enhanced indicating the detection system is functional.

The IFN antagonist activities of the V proteins were further detected with the luciferase assay system. Cells were co-transfected with pChIFN-β-luc 250 ng, pRL-TK 10 ng and pCMV-3HA-V 250 ng (or pCMV-3HA). And then the cells were stimulated with poly(I:C) at 24 h post-transfection. The samples were collected at 48 h post-stimulation (hps), and the expressions of the luciferase were quantified with luciferase detection kit. Higher level of luciferase activities was detected in those cells transfected with empty vector pCMV-3HA (Fig. 2a). V proteins caused varying degree of IFN antagonism as represented by different levels of IFN-β expression. Those V proteins from VG NDVs caused the most significant decrease in luciferase production followed by the V proteins from MG NDV strains. The interferon

Table 1 Information of these V proteins and their corresponding virus

Isolates	Host	Year	Genotype	Virulence	MDT	GenBank.No
Shaanxi06 [2]	Crested Ibis	2006	VIId	VG	54.4	KC954584
Shaanxi10 [2]	Crested Ibis	2010	VIi	MG	84.4	KC954585
HX01 [3]	Swine	2009	II	LG	91.2	KC954586
Gfw-10	Grey-faced woodpecker	2010	Vib	MG	78 (detected by our lab)	KC954593
sd-08	Spotted dove	2008	IX	VG	52 (detected by our lab)	KC954591
F48E9 [9]	Chicken	1948	IX	VG	44	KC954598

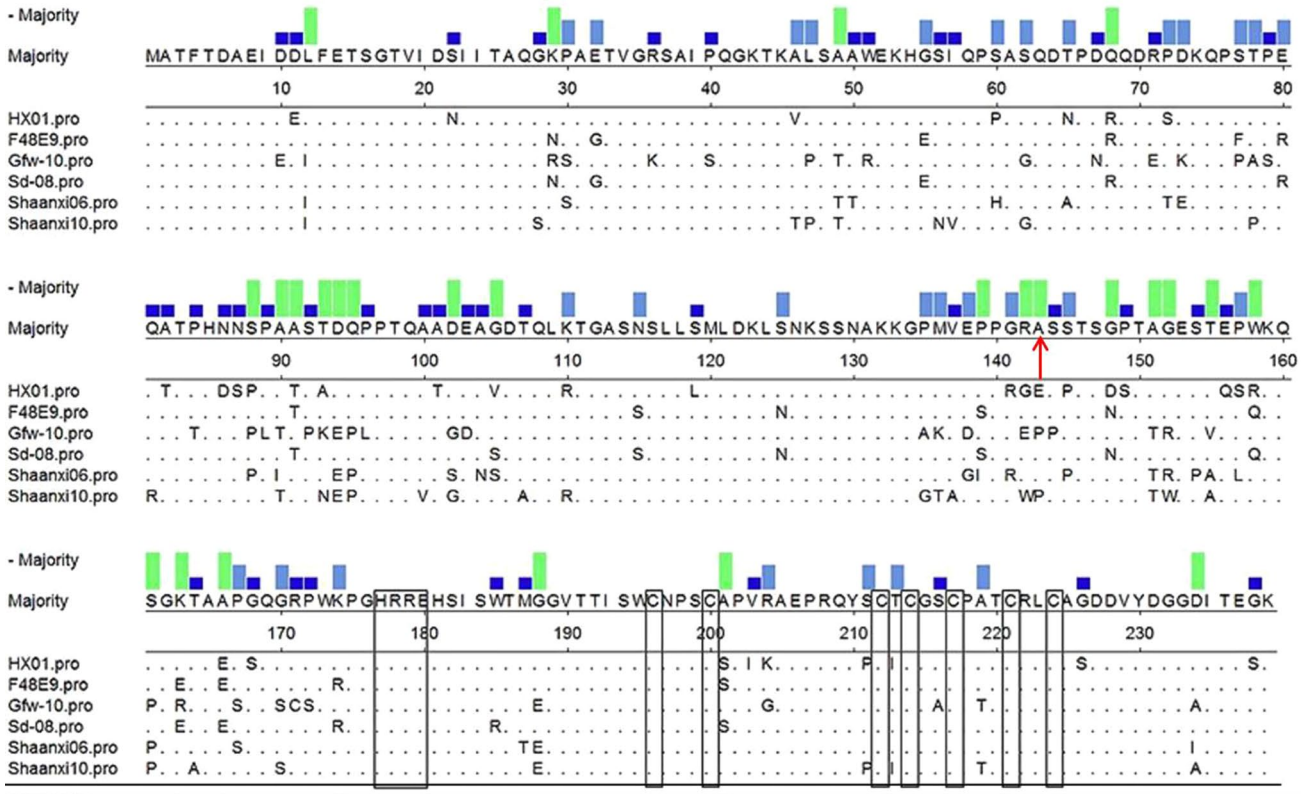

Fig. 1 Polygenetic alignment analysis of the amino acids sequences of V proteins used in the current study. The boxes were used to show the amino acids forming the conserved zinc finger structure at C terminal of V proteins

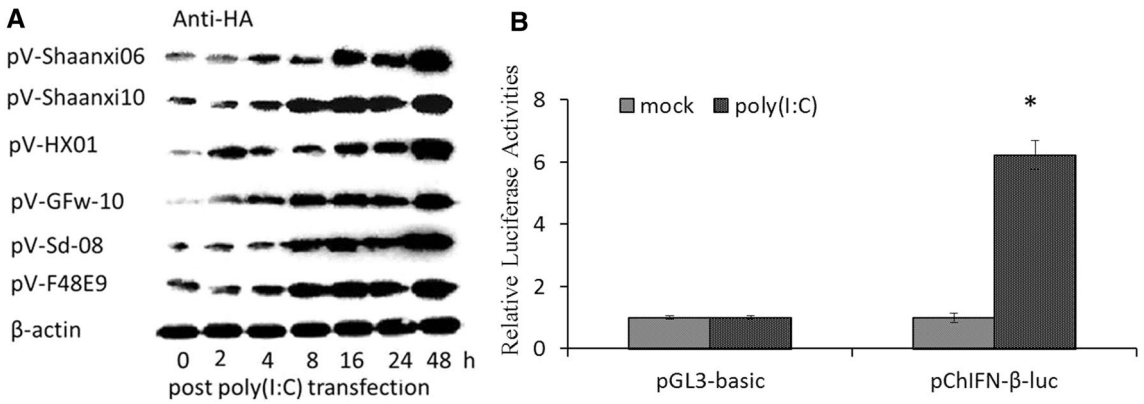

Fig. 2 Western blotting detection V proteins expression at various time points after transfection (**a**) and luciferase assay detect IFN-β promoter activation (**b**)

antagonistic activities of V proteins from LG NDV strains were the lowest (Fig. 3a). IFN-β expression in supernatants after transfection with plasmid pCMV-3HA vectors/V protein expression vectors and poly(I:C) were also quantified with an ELISA kit (mlbio, Shanghai). The cell culture supernatants were collected at 0, 2, 4, 8, 16, 24, and 48 h post-poly(I:C) treatment and the concentrations of IFN-β in those samples were quantified. The results showed that IFN-β expressions increased slowly within 16 hps. And then, IFN-β expressions sharply increased during 16 to 24 hps (Fig. 3c) in all groups. The significant differences of IFN-β expression between each group were observed at 48 hps (Fig. 3c, d). V protein derived from VG NDVs reduced about 80% IFN-β expression compared with that of Poly

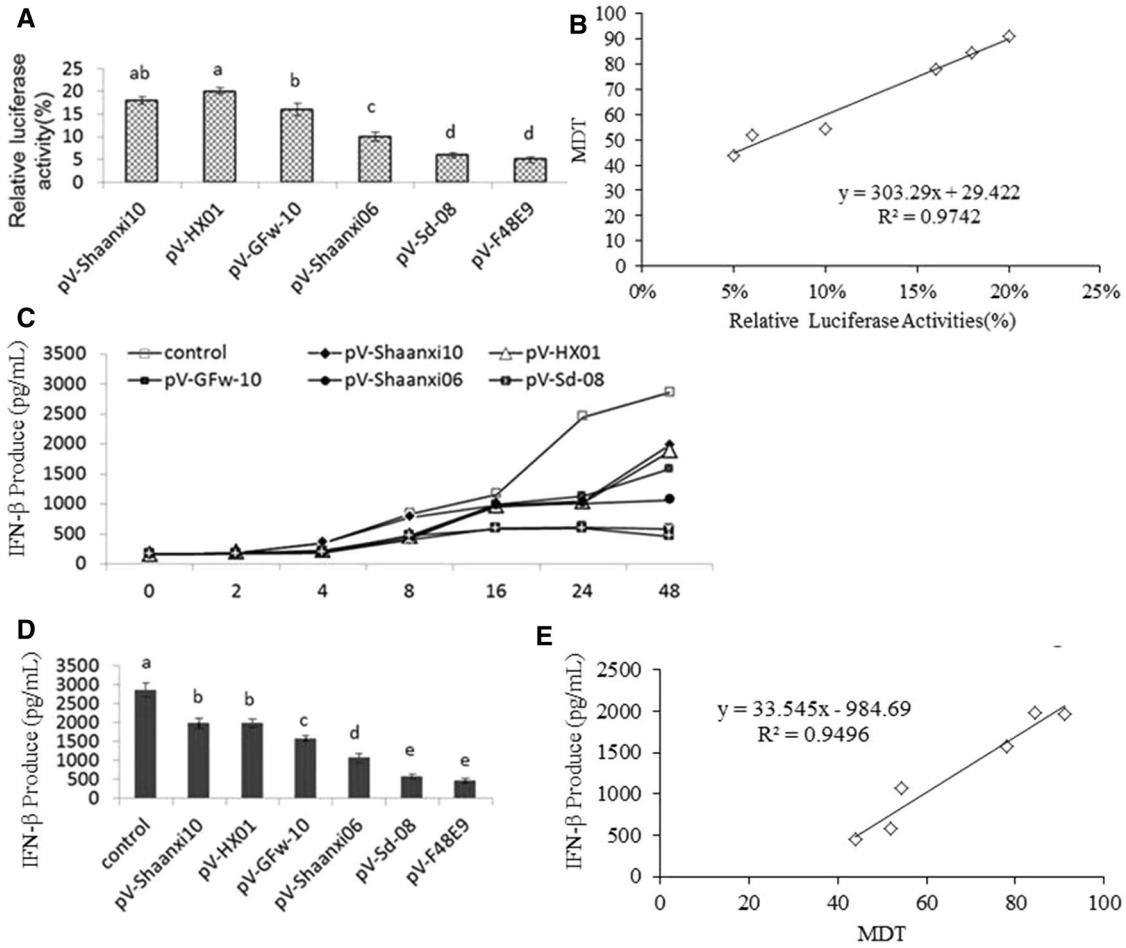

Fig. 3 V proteins showed different abilities on restriction IFN-β expression. Luciferase assay was used to detected luciferase activities in cells transfected with empty plasmid (control) or plasmids harboring different V genes and poly(I:C). Samples were collected at 48 hps, and the data are showed as a relative percentage to control (**a**). ELISA was used to detect IFN-β expression in supernatant collected at 0, 2, 4, 8, 16, 24, and 48 h post-poly(I:C) treatment (**c**), and significant differences were observed at 48 h (**c**). Correlation analyses indicated a good collection between virulent and their V protein activity (**b, e**)

(I:C) together with empty vectors induced IFN-β expression (control). And V proteins derived from MG NDVs decreased about 40% IFN-β expression (Fig. 3d).

Correlation analysis was used to understand the relationship between IFN-β expression and viral virulence index, MDT. As shown in Fig. 3b, e, both the ELISA results and luciferase assay results had good correlation with virus MDT index as the correlation indexes, R^2, were all greater than 0.9.

The amino acid sequence alignment show that the V proteins shared 60–90% homology and the V proteins derived from same NDV pathotype have higher homology. As reported previously, paramyxovirus V proteins have highly conserved C-terminal domain (CTD) [10]. And one invariant histidine and 6 cysteines in CTD forms a unique zinc finger fold. These amino acids forming the zinc finger play important role in antagonizing IFN and mutations in those amino acids lead to the loss of the V proteins function [10]. In addition, the first arginine (R), glutamic acid (E) and isoleucine (I) are important to maintain the function of V protein. NDV V protein shared similar structure in CTD with other paramyxovirus V proteins and all of these six V proteins used showed same amino acids at those sites (Fig. 1). The other four sites (144,153,161 and 234) that have been identified for playing roles in regulating the function of V protein have also been analyzed previously [1]. However, no distinct roles were found at those sites, as VG strains may use same amino acids as LG or MG NDV strains at those sites. However, at site 143, amino acids E, proline (P) and Alanine (A) were used by LG, MG and VG NDV strains, respectively. Whether this site plays a role in determination the function of V protein needs further research to reveal.

The mechanism by which the NDV V protein antagonizes IFN has been studied previously. By creating a recombinant

virus with a carboxyl-terminus truncated V protein (rBC/V-stop) and a recombinant virus, Huang et al. (2003) proved that V protein could induce the degradation of STAT1 [6], one important molecule in IFN pathway. Later, V protein derived from La Sota strain of NDV, as well as 12 other paramyxoviruses was found to interact with the RNA helicase, MDA-5 via the conserved cysteine-rich CTD [4]. Plasmids encoding V proteins inhibited IFN-β promoter activity were also identified with an IFN-β promoter reporter system [4]. While, as all the V proteins have the same core elements of CTD, the mechanism of different IFN anatomization activities of different NDV pathotypes needs further researches to reveal.

Here, we find a good correlation between viral MDT and in vitro IFN promoter activity and IFN-β production. The only way to identify the exact role of each amino acid residue in the CTD of the NDV V proteins of different NDV pathotypes in interferon antagonism is to generate and test specific recombinant V protein mutant viruses, as NDV virulence is determined by multiple genes [5].

In summary, we identified a good correlation between viral virulence and interferon antagonist activity of the corresponding V protein using six NDV strains with known pathogenic type. And according to sequence analysis, several other amino acid residues may be related to interferon antagonistic activities of protein V besides the conserved zinc finger structure.

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Author contributions XW performed the experiments and made analysis of the data. RD and ZY participated preparation including discussion and editing.

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Data Availability All data generated or analyzed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interests.

Informed consent All the authors consent to publish.

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