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Antigenic validation of recombinant hemagglutinin-neuraminidase protein of Newcastle disease virus expressed in *Saccharomyces cerevisiae*

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Summary. – The outer membrane glycoprotein, hemagglutinin-neuraminidase (HN) of Newcastle disease virus (NDV) is important for virus infection and subsequent immune response by host, and offers target for development of recombinant antigen-based immunoassays and subunit vaccines. In this study, the expression of HN protein of NDV is attempted in yeast expression system. Yeast offers eukaryotic environment for protein processing and posttranslational modifications like glycosylation, in addition to higher growth rate and easy genetic manipulation. *Saccharomyces cerevisiae* was found to be better expression system for HN protein than *Pichia pastoris* as determined by codon usage analysis. The complete coding sequence of HN gene was amplified with the histidine tag, cloned in pESC-URA under GAL10 promotor and transformed in *Saccharomyces cerevisiae*. The recombinant HN (rHN) protein was characterized by western blot, showing glycosylation heterogeneity as observed with other eukaryotic expression systems. The recombinant protein was purified by affinity column purification. The protein could be further used as subunit vaccine.

Keywords: Newcastle disease virus; glycoprotein; codon usage; yeast expression

Introduction

Newcastle disease (ND) is a severe respiratory disease affecting the farmed poultry and free-living birds and is a constant threat to the commercial chicken industry around the world. Special emphasis is being put forth to prevent the spread of ND by efficient biosecurity and flock management practices, development of rapid diagnostics, novel antiviral therapy and vaccination strategies (Alexander, 2008). Traditionally, antigens associated with pathogenic organisms like ND virus (NDV), are produced for the development of immunoassays and vaccines by inoculation of embryonated chicken eggs or infecting susceptible cultured cells with the virus followed by purification and inactivation of the virus or extraction of viral antigens. Due to potential biosecurity hazards and handling of potential pathogens, recombinant proteins have become popular as antigens to replace the traditionally inactivated viruses (Wang *et al*., 2006). With the development of recombinant DNA technologies, various expression systems such as bacterial, yeast, insect and mammalian hosts are available for this purpose.

The hemagglutinin-neuraminidase (HN) protein of NDV is present as outer membrane spikes and is responsible for virus attachment and host immune response. Hence, the HN protein has been explored for the development of rapid antigen detection kit and subunit vaccines. Mohan *et al*. (2006) expressed the HN protein of NDV in *Escherichia coli*, a typical prokaryotic expression system. The prokaryotic host does not offer any posttranslational glycosylation of proteins. However, the recombinant HN (rHN) protein expressed in *E. coli* was antigenically functional in an ELISA format. The HN protein of NDV is immunologically important as it also induces specific neutralizing antibody response (Schaper *et al*., 1988) and thus has a vaccination potential (Long *et al*.,

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Abbreviations: CUSP = create codon usage table program; Emboss = European Molecular Biology Open Software Suit; HN = hemagglutinin-neuraminidase; ND = Newcastle disease; NDV = ND virus; rHN = recombinant HN; RSCU = relative synonymous codon usage value

1986). Since, HN is present naturally in glycosylated form, and *E. coli* has no glycosylation pathway for processing nascent polypeptides, the recombinant protein often fails to fold properly, hampering the activity and yield, an important determinant towards development of a subunit vaccine.

The eukaryotic expression of HN protein mediated through baculovirus has been widely used, as insect cells have the ability for protein folding and post-translational modifications (Nagy *et al*., 1991; Niikura *et al*., 1991; Ong *et al*., 1999; Lee *et al*., 2008). But the failure to achieve high cell densities limited the full exploitation of the potential productive capacity of the baculovirus expression system (Radford *et al*., 1997). The heterologous expression of protein in mammalian system produces recombinant protein in native conformation but is expensive and has lower production level. Use of avian cells for the expression of NDV HN glycoprotein for immunization has been reported earlier (Cosset *et al*., 1991). Further, the full length HN gene has also been cloned into a mammalian expression vector and characterized for transient expression of protein in cell culture system by Sawant *et al.* (2011). In contrast, the yeast cells being single celled, has the ability for fast growth, amenable to genetic manipulation and possesses eukaryotic features including a secretory pathway leading to correct protein processing and post-translational modifications (Mattanovich *et al*., 2012).

Expression of the complete coding sequence of HN gene of NDV has not been reported in yeast. King (2004) described the absence of HN protein expression in *Pichia pastoris* and *S. cerevisiae* cells, however, reported the expression of HN protein as an insoluble inclusion body in *E. coli*. A secreted form of HN protein, which lacked the regions encoding the cytoplasmic tail and the transmembrane anchor sequences, has been expressed in *P. pastoris* and secreted into the culture supernatant (Peeters *et al*., 2001). This yeast culture supernatant has been characterized by HN-specific monoclonal antibodies in capture ELISA using a limited number of serum samples with low titer, thereby demanding further optimization and validation of the test (Peeters *et al*., 2001). Hence, the expression profile, glycosylation pattern and antigenic nature of HN obtained from yeast expression system could not be determined.

Each expression host used in recombinant protein production has unique codon usage pattern that determines the level and quality of a heterologous gene expression. In this study**,** we report the *in-silico* analysis of codon usage preference for HN gene of NDV in *S. cerevisiae* and *P. pastoris*, the two routinely used yeast expression systems and subsequent expression and characterization of HN gene from the most suitable yeast expression host.

Materials and Methods

Selection of suitable yeast system by synonymous codon usage analysis. To select most suitable yeast expression system for rHN protein production, *in-silico* comparison was done for codon usage biasness among *S. cerevisiae* and *P. pastoris.* The relative synonymous codon usage value (RSCU) determines peculiarity of codon usage and was calculated with CUSP tool (create codon usage table program) of European Molecular Biology Open Software Suit (Emboss) (Table 1). The RSCU value <1.0 indicates negative codon bias, i.e less abundant codon, and >1.0 indicates positive codon bias,

Table 1. Synonymous codon usage of NDV HN gene analyzed with CUSP

Sr. No.	Codon	Amino acid	[a]Fraction	[b]Frequency	[c]Number	[d]RSCU
1	AAA	K	0.5	19.031	11	1
2	AAC	N	0.44	19.031	11	0.88
3	AAG	K	0.5	19.031	11	1
4	AAT	N	0.56	24.221	14	1.12
5	ACA	T	0.538	48.443	28	2.15
6	ACC	T	0.173	15.571	9	0.69
7	ACG	T	0.019	1.73	1	0.08
8	ACT	T	0.269	24.221	14	1.08
9	AGA	R	0.235	13.841	8	1.41
10	AGC	S	0.164	17.301	10	0.98
11	AGG	R	0.206	12.111	7	1.24
12	AGT	S	0.148	15.571	9	0.89
13	ATA	I	0.342	22.491	13	1.03
14	ATC	I	0.395	25.952	15	1.18
15	ATG	M	1	17.301	10	–
16	ATT	I	0.263	17.301	10	0.79
17	CAA	Q	0.4	10.381	6	0.8
18	CAC	H	0.333	6.92	4	0.67
19	CAG	Q	0.6	15.571	9	1.2

Table 1. (continued)

Sr. No.	Codon	Amino acid	[a]Fraction	[b]Frequency	[c]Number	[d]RSCU
20	CAT	H	0.667	13.841	8	1.33
21	CCA	P	0.233	12.111	7	0.93
22	CCC	P	0.333	17.301	10	1.33
23	CCG	P	0.167	8.651	5	0.67
24	CCT	P	0.267	13.841	8	1.07
25	CGA	R	0.176	10.381	6	1.06
26	CGC	R	0.176	10.381	6	1.06
27	CGG	R	0.176	10.381	6	1.06
28	CGT	R	0.029	1.73	1	0.18
29	CTA	L	0.13	10.381	6	0.78
30	CTC	L	0.174	13.841	8	1.04
31	CTG	L	0.109	8.651	5	0.65
32	CTT	L	0.174	13.841	8	1.04
33	GAA	E	0.565	22.491	13	1.13
34	GAC	D	0.414	20.761	12	0.83
35	GAG	E	0.435	17.301	10	0.87
36	GAT	D	0.586	29.412	17	1.17
37	GCA	A	0.417	25.952	15	1.67
38	GCC	A	0.222	13.841	8	0.89
39	GCG	A	0.167	10.381	6	0.67
40	GCT	A	0.194	12.111	7	0.78
41	GGA	G	0.205	13.841	8	0.82
42	GGC	G	0.128	8.651	5	0.51
43	GGG	G	0.385	25.952	15	1.54
44	GGT	G	0.282	19.031	11	1.13
45	GTA	V	0.39	27.682	16	1.56
46	GTC	V	0.195	13.841	8	0.78
47	GTG	V	0.244	17.301	10	0.98
48	GTT	V	0.171	12.111	7	0.68
49	TAC	Y	0.385	17.301	10	0.77
50	TAT	Y	0.615	27.682	16	1.23
51	TCA	S	0.213	22.491	13	1.28
52	TCC	S	0.082	8.651	5	0.49
53	TCG	S	0.066	6.92	4	0.39
54	TCT	S	0.328	34.602	20	1.97
55	TGC	C	0.583	12.111	7	1.17
56	TGG	W	1	6.92	4	-
57	TGT	C	0.417	8.651	5	0.83
58	TTA	L	0.283	22.491	13	1.7
59	TTC	F	0.591	22.491	13	1.18
60	TTG	L	0.13	10.381	6	0.78
61	TTT	F	0.409	15.571	9	0.82
62	TAA	*	0	0	0	-
63	TAG	*	1	1.73	1	-
64	TGA	*	0	0	0	-

Footnote: [a]Fraction column shows the proportion of each of the synonymous codons (encoding the same amino acid). [b]Frequency/1000 value represents the number of codons percent per 1000 bases in the input sequence. [c]Number represents the number of occurrences of each codon. [d]RSCU value is used to determine the relative synonymous codon usage.

i.e abundant codons, whereas RSCU value = 1.0 denotes random or equal usage of codon.

Codon preference analysis. Codon preferences of NDV HN gene were compared for ratio of codon usage frequency (1/1000) for *S. cerevisiae* and *P. pastoris* (obtained from http://www.kazusa.or.jp/codon/) to evaluate the suitable host for the optimal expression of HN protein in host (Fig. 1). The ratio of usage preference >2.0 and <0.5 shows distinct usage differences between NDV and any

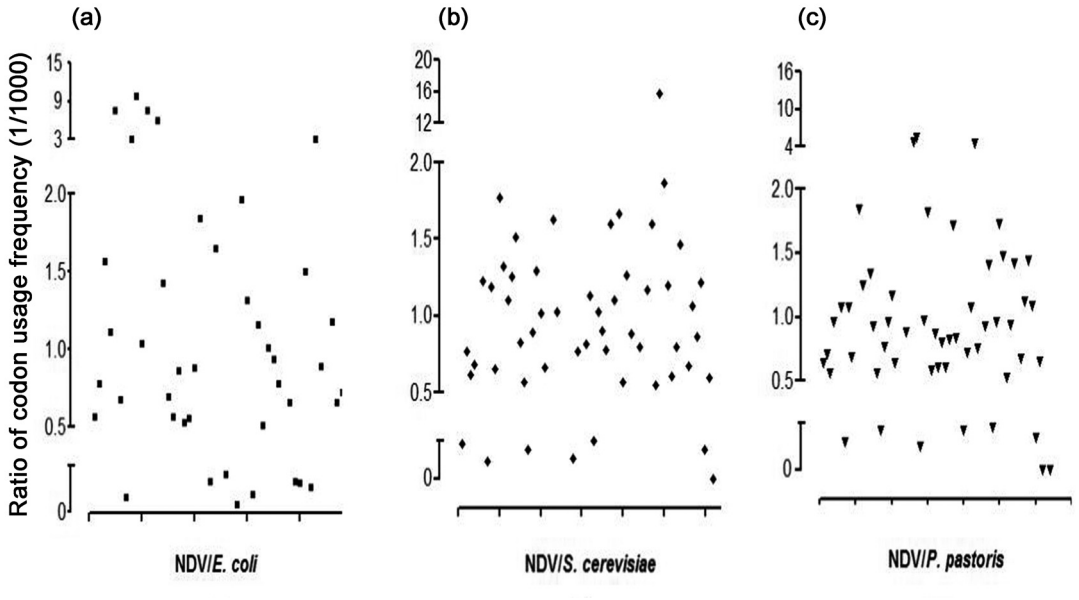

Fig. 1

Comparison of the ratio of codon usage frequency (1/1000) of NDV-HN gene to (a) *E. coli* (b) *S. cerevisiae* (c) *P. pastoris*
Ratio higher than 0.5 and lower than 2 indicates similar codon usage preference.

heterologous expression system, whereas ratio between 0.5 and 2.0 denotes similar codon usage preference between the two systems.

Virus. The mesogenic NDV strain isolated from wild peacock characterized previously was used in this study (Khulape *et al*., 2014). The virus was maintained in the Recombinant DNA Laboratory, Indian Veterinary Research Institute. The virus was cultivated in the allantoic cavity of specific pathogen free 9-day-old embryonated chicken eggs and the infected allantoic fluid collected after 72 hr of infection was used for RNA extraction.

Viral RNA extraction and RT-PCR. The RNA was extracted from allantoic fluid infected with NDV isolate using TRIZOL®LS reagent as per manufacturer's instructions. The cDNA synthesis was performed using random hexamer primer with a SuperScript®III first strand synthesis kit as per manufacturer's instructions (Invitrogen, USA).

Amplification and cloning of the HN gene. The complete coding sequence (CDS) of HN gene was amplified from cDNA by Taq DNA polymerase (Thermo Scientific, USA) using forward (HN-NotI-5'-CCGCGGCCGCATGGACCGCGCAGTTAGC-3') and reverse (HN-BglII-5'-CCAGATCTCTAATGATGATGATGATGATGACCAGACCTGGCTTCTCTAAC-3') primers with in-built restriction enzyme sites. The amplification was performed at an initial denaturation of 94°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 55°C for 60 sec, 72°C for 1 min and final extension of 72°C for 10 min. The amplified product was cloned in pTZ57R /T vector (Thermo Scietific, USA) following manufacturer's instructions. The HN cds was further subcloned into pESC-URA expression vector (Stratagene, La Jolla, CA) downstream of the GAL10 promoter and named as pESC-URA-HN. The integrity of the plasmid at cloning and sub-cloning step was verified by restriction enzyme analysis and sequencing.

Expression and characterization of HN gene. The pESC-URA-HN plasmid was transformed into *S. cerevisiae* strain YH501 (Stratagene, La Jolla, CA) as described previously (Dey *et al*., 2009). The recombinant yeast clones were selected on auxotrophic SG-ura medium containing galactose, yeast extract without amino acids and amino acid drop out media (without uracil). The colonies were characterized for rHN protein expression by western blot assay. The blots were probed with in house chicken anti-NDV polyclonal antibody and detected with rabbit anti-chicken secondary antibody conjugated to HRP (Sigma, USA).

Results

Selection of yeast expression host

The RSCU analysis of HN gene (Table 1) of NDV showed that out of 61 codons, 29 (47.5%) were used less abundantly, whereas 28 (45.9%) codons were favored more frequently and only two codons were shown to be used randomly for expression of functional HN gene. Also, 45 and 42 number of codons with similar usage pattern were found when the expression of NDV HN protein was considered in *S. cerevisiae* and *P. pastoris,* respectively. This indicated that the codon usage of HN protein was more closely related to *S.*

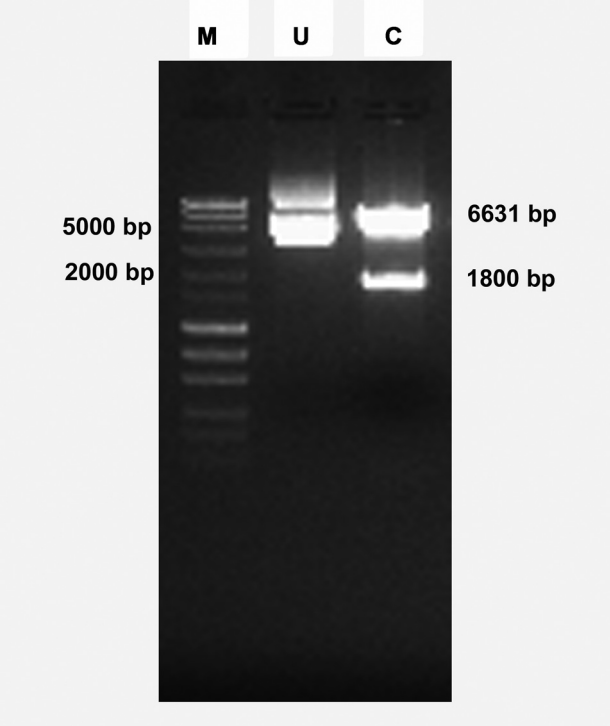

Fig. 2

Restriction enzyme screening of plasmid pESC-URA-HN
Lane M: DNA molecular weight ladder; Lane U: Uncut pESC-URA-HN construct; Lane C: pESC-URA-HN construct following digestion with *Not*I and *Bgl*II released an insert of the size of HN gene (~1800 bp) and pESC-URA vector (6631 bp).

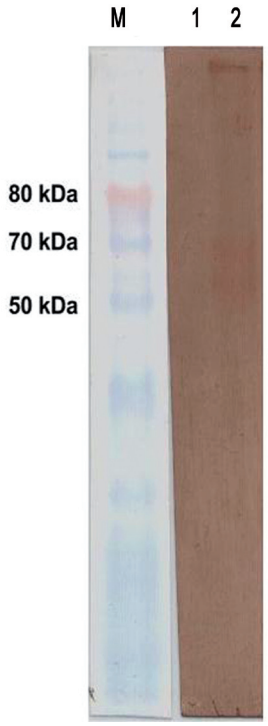

Fig. 3

Recombinant HN protein detected by Western blot assay
Lane M: Prestained marker; Lane 1: Empty pESC-URA vector transformed in yeast cells; Lane 2: Yeast cell lysate transformed with pESC-URA-HN plasmid.

cerevisiae than to *P. pastoris.* Further, it also revealed that *S. cerevisiae* can compensate for the codon usage pattern more effectively than *P. pastoris*.

Amplification and cloning of HN gene

To subclone the NDV HN gene, a specific amplicon of the size of approximately 1800 bp was obtained by PCR, as expected. The amplified product was cloned in pTZ57R/T vector and subcloned into the pESC-URA vector. The restriction enzyme analysis of pESC-URA-HN using *Not*I and *Bgl*II showed specific release of HN cds (Fig. 2).

Expression of HN protein in S. cerevisiae

Western blot analysis of yeast cell lysate showed that rHN protein reacted specifically as a double band pattern with the molecular weight between 50 kDa and 80kDa when probed with polyclonal sera against NDV (Fig. 3). The banding pattern obtained for rHN protein corresponds to differential glycosylation extent in the yeast cell.

Discussion

Recombinant antigens derived from various expression systems have become integral part for development of immunoassays and vaccines, overcoming the shortfalls of traditional antigen preparation protocols with respect to quality, convenience and time. We report the successful expression of complete HN gene of NDV in *S. cerevisiae,* a yeast expression system. As an outer membrane glycoprotein, NDV HN plays a pivotal role in infection and immunity of the host. Sialic acid receptors on host cells are recognized by HN protein that leads to the virus attachment (Scheid and Choppin, 1973). Additional neuraminidase activity of HN protein helps in hydrolysis of progeny's sialic acid to prevent self-agglutination. HN protein is necessary for penetration of the virus into the cell and determines the tropism and virulence of the virus (Huang *et al*., 2004). Thus, HN protein has been targeted for the development of recombinant-antigen based diagnostics and subunit vaccines.

The HN protein of NDV has been previously expressed in a common prokaryotic expression system *E. coli* (Mo-

han *et al.*, 2006). The recombinant protein obtained from prokaryotic host was compromised for glycosylation, activity and yield, thus has an undesirable property towards production of a subunit vaccine. A well-qualified baculovirus-based eukaryotic system has been also explored for production of rHN protein but the utility was hindered due to the failure to achieve high cell densities, limiting the potential productive capacity of the host (Radford *et al.*, 1997).

The yeast expression of complete HN gene has been attempted earlier but the expression profile, glycosylation pattern and antigenic nature of rHN protein could not be determined (King, 2004). The study was carried out to express the complete HN gene in a yeast. *S. cerevisiae* and *P. pastoris*, the two commonly used yeast hosts were compared *in-silico* to select the best suitable system for expression of HN gene based on synonymous codon usage analysis. This codon usage preference varies with the species and determines the degree of heterologous gene expression in the host (Wang *et al.*, 2011). Additionally, this specific inclination for particular codon usage is essential in understanding the evolution of pathogen and subsequently for the expression of immunogenic proteins in the most desirable expression system. Both mutational bias and natural selection determine the codon usage pattern of a particular gene and in case of NDV, the mutational pressure is considered as the main factor (Wang *et al.*, 2011).

The codon usage of HN protein was found to be more closely related to that of *S. cerevisiae* than to that of *P. pastoris*. Hence, we speculated that *S. cerevisiae* could be a better expression system than *P. pastoris* with wide range of strains available, together with eliminating the problem of methanol toxicity as observed during induction of *P. pastoris*. The antigenic analysis based on Western blot of yeast lysate showed specific reactivity of rHN protein. The banding pattern obtained for rHN protein corresponds to differential glycosylation extent in yeast cell. The glycosylation heterogeneity for rHN protein has previously been reported following expression in an insect cell line using baculovirus vector system (Zoth *et al.*, 2011).

To the best of our knowledge, this is the first report wherein the complete cds of HN gene was expressed in the yeast *S. cerevisiae*. The yeast cells were able to generate glycosylated HN protein with proper folding and antigenicity. Since the HN expressed in this study was also tagged with 6× histidine, Ni-NTA column was used for affinity purification of the protein. This allowed the protein to be obtained in a pure form, which can be further employed for use as subunit vaccine or as an antigen-based diagnostic test. The present study validates yet another system for efficient expression of HN that could also be explored for expression of other outer membrane glycoprotein of NDV.

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