JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov. 2013. Vol. **7**(Spl. Edn.), p. 727-733

Optimization of Infectious Bronchitis Virus Nucleocapsid Protein's *in-vitro* Expression using Response Surface Methodology

Zhang De-Yong[1]*, Xu Xiao-Lu[1], Shen Xiu-Ying[2], Lu Yin[1], Xu Hui-Ying[1] and Hao Fei-Lin[1]

[a]College of Biology and Environmental Engineering, Zhejiang Shuren University, Hangzhou, China
[b]College of Biology and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou, China.

(Received: 27 September 2013; accepted: 04 November 2013)

Nucleocapsid protein(N) of avian coronavirus infectious bronchitis virus(IBV) plays an important role in maintaining viral structure and replication. The N protein produced in vitro can be applied in the fields of diagnosis, vaccination and scientific research. Expression of IBV N protein in a prokaryotic expression system has been established before, but the medium used is complex and expensive. To optimize the fermentation conditions with simpler medium, the Plackett-Burman experiment was designed for factor screening, and then response surface methodology was applied to optimize the fermentation conditions. The medium was optimized as 0.86 % polypeptone, 1.14 % yeast extract, 1.63 % NaCl, 0.02 % L-arabinose and pH 7.0. Five batches of fermentation according to the above conditions resulted in a mean value of 44.25% for the relative N protein quantity, with a 0.14 % deviation from theoretically deduced value. The fermentation conditions optimized in this study is appropriate for N protein production, which stablely expresses IBV N protein and gives a high yield.

Key words: IBV; N gene; Expression *in vitro*; Plackett-Burman experiment; Response surface methodology.

Avian coronavirus infectious bronchitis virus (IBV) usually causes infectious bronchitis (IB), which is an acute and highly contagious disease, occurring worldwide and resulting in severe economic loss in the poultry industry[1,2]. Attenuated live vaccines are used widely to control IB, but like most coronavirus, IBV varies frequently and currently exists as many serotypes. Different tissue tropisms were even observed for these variant strains[3]. The genome of IBV encodes three major structural proteins: spike protein (S), membrane protein(M) and nucleocapsid protein (N). S protein carries serotypespecific sequences and neutralization epitopes and it is highly involved in infectivity. N protein is a phosphorylated protein, which is constituted of 409 amino acids and has a molecular weight of about 50KD[2].

For positive strand RNA viruses, N protein usually plays a key role in virus biology, involved in encapsidation, assembly and regulation of viral RNA synthesis[4-7]. These proteins may also interact with host cell proteins to facilitate their activities related to the virus life cycle, but the interaction possibly disrupt host cell signaling pathways[8]. N protein of IBV also has multiple

* To whom all correspondence should be addressed.
Tel: +86-571-88298514; Fax: +86-571-88297098;
E-mail: deyonghz@163.com

functions in the virus life cycle. By binding to the genome located inside a virion, N protein is critical for maintaining the structure of IBV and controlling its replication and assembly[9]. N protein is produced early and abundantly during virus infection (the molar ratio of N/S is about 6/1)[10]. Koch *et al*. confirmed that some antibodies against N protein also showed also neutralization activity[11]. Boots *et al.* found that the expressed N protein in DNA immunity experiment could induce immune response against the whole virion in mouse and in chicken, with activation of Th cell and B cells[12].

Generally, N protein is expressed abundantly in host animals after vaccination or infection. The antibody titre against N protein is high and is easy to be detected. Moreover, different from S protein, N protein is highly conserved among different IBV strains[13]. Therefore, N protein is an appropriate immunodiagnostic molecule for serum antibody evaluation in vaccinated flocks or infected flocks. A traditional way to prepare virus's structural protein is purifying the virus from host cells and then to separate the aimed proteins directly, which is exhausting and inefficient. Now it is easy to express virus structural proteins *in vitro* via genetic engineering. Our previous work generated a way to express N protein in recombinant *Escherichia coli* and established an ELISA assay based on the expressed N protein[14].

To achieve efficient expression of N protein *in vitro* and to reduce the fermentation costs, this study aims to optimize the fermentation conditions by response surface methodology (RSM), an efficient method using designed experiments to optimize multiple factors interacted.

MATERIALS AND METHODS

Construction of recombinant Escherichia coli for N protein expression

The detailed construction operation of recombinant *E. coli* for N protein expression was described in our previous study[14]. Briefly, the coding region of the N gene of IBV isolate X(GenBank No. AY043221, AY043315), 1230bp in length, was cloned and ligated into the expression vector pBAD/his B (Invitrogen Co., CA, USA). The recombinant vector pBAD/his B-N was then transformed into *E. coli* strain LM194 (Invitrogen Co., CA, USA) for expression *in vitro*. It is necessary to point out that, this prokaryotic expression system uses L-arobose as inductor.

Quantification of N protein

N protein was identified by western blotting as described in our previous study[14]. Quantification of N protein was performed by SDS-PAGE electrophoresis and subsequent image quantification analysis which was based on comparing the density of the protein bands. For each group, 1.5 mL bacterial culture was sampled for analysis. Bacterial pellets were collected by centrifugation, and were then resuspended in 100 ¼L 1× loading buffer and boiled for 10 min. After a centrifugation, 10 ¼L supernatant was taken and loaded in a 12% SDS-PAGE gel. Based on the image analysis using Biorad Geldoc XR system, the ratio of N protein to the total bacterial proteins in each lane was taken as an indicator to evaluate expression efficiency.

Optimization of fermentation time by the single factor experiment

The recombinant *E. coli* was cultured in LB medium to an OD_{600} value of 0.8 and was then induced with 0.02% final concentration of L-arabinose for N protein expression. The culture was sampled at 1h, 2h, 3h, 4h and 5h after induction respectively for N protein quantification. N protein was separated by a 12% SDS-PAGE gel, and its expression was relatively quantified by image analysis using the Biorad Geldoc XR system (Bio-Rad company, USA).

Optimization of fermentation conditions by response surface methodology

Factor screening by the Plackett-Burman design method

Plackett-Burman(PB) design aims to screen important factors, which have most significant effect on results, to simplify the subsequent experiment design. As shown in Table 1 and Table 2, two levels were designed for each factor. The designed low level for tryptone, yeast extract, NaCl, pH, Ampicilin, L-arabinose and inoculation ratio of seeds was 1%, 0.5%, 1%, 7.0, 0.05g/L, 0.02% and 1% respectively. The designed high level for the above components was 1.5%, 0.75%, 1.5%, 8.5, 0.075g/L, 0.3%, and 1.5% respectively. In addition, four virtual factors(V_3, V_5, V_8 and V_{11} in Table 1) were also designed for error evaluation. Based on SDS-PAGE image

analysis, the ratio of N protein to total bacterial proteins in each lane was taken as the relative quantity of N protein. Statistical significance was analyzed using variance analysis. For PB experiment, significance was defined as $p<0.1$.

Level determination by the steepest ascent experiment

After the three main factors were determined using the PB experiment, a steepest ascent experiment was designed to choose appropriate varying ranges for them in the following RSM. All of the three factors were designed FOR a varying range at 0.3 intervals. The range for tryptone, yeast extract and NaCl was 0.3 to 1.5(%), 0.5 to 1.7(%) and 1.0 to 2.2% respectively. In total, five experiments were performed.

Optimization of fermentation medium by Box-behnken response surface methodology

As shown in Table 3, three levels for each factor were designed for optimization of fermentation conditions based on their central concentration level. The concentration of tryptone varied from 0.3 % to 1.5 %. The concentration of yeast extract varied from 0.5 % to 1.7 %. The concentration of NaCl varied from 1.0 % to 2.2 %. Totally 15 experiments were completed using RSM. The optimized fermentation conditions was confirmed by 5 repetitive experiments for variance anaysis. Statistical significance was defined as $p<0.05$.

RESULTS

The Plackett-Burman experiment

The relative N protein quantity varied from 24.7% to 44.1% in Plackett-Burman experiment. Three main factors (tryptone, NaCl, and yeast extract) were found to affect fermentation results obviously ($p<0.1$). The order for their importance was tryptone>NaCl>yeast extract (Table 1 and Table 2).

The steepest ascent experiment

The impact coefficient of tryptone, yeast extract and NaCl was confirmed to be negative, positive and positive, respectively. Therefore, the stepping method for them was decided to be degression, increment, and increment respectively. The relative quantity of N protein in the 5 experiments was each 39.1041.7042.3042.4 and 45.1. A step size of 0.3 was thought to be appropriate for all of the three main factors(Fig. 1).

Response surface methodology

Based on the individual central level, three levels (high level, central level, and low levels) were designed for each factor(Table 3). Data analysis was then carried out using the response surface regression method in Design-expert 7.0, and a quadratic response surface regression model was established. The equation was as follows:

$$Y=44.24-0.59X_1+0.72X_2+0.99X_3-1.75X_1X_2+0.88X_1X_3-2.20X_2X_3-4.61X_1X_1-4.95X_2X_2-7.35X_3X_3.\ (R^2=0.9517, \text{Regression SD}=2.85)$$

Table 1. The design and results of Plackett-Burman experiment

Try (%)	Yea (%)	V_3	NaCl (%)	V_5	pH	Amp (g/L)	V_8	Ara (%)	Ino (%)	V_{11}	N (%)
1.5	0.5	1	1.0	-1	7.0	0.075	1	0.03	1.0	1	24.7
1.5	0.75	-1	1.5	-1	7.0	0.05	1	0.03	1.5	-1	40.2
1.0	0.75	1	1.0	1	7.0	0.05	-1	0.03	1.5	1	40.0
1.5	0.5	1	1.5	-1	8.5	0.05	-1	0.02	1.5	1	36.5
1.5	0.75	-1	1.0	1	7.0	0.075	-1	0.02	1.0	1	35.4
1.5	0.75	1	1.0	1	8.5	0.05	1	0.02	1.0	-1	35.3
1.0	0.75	1	1.5	-1	8.5	0.075	-1	0.03	1.0	-1	44.1
1.0	0.5	1	1.5	1	7.0	0.075	1	0.02	1.5	-1	41.5
1.0	0.5	-1	1.5	1	8.5	0.05	1	0.03	1.0	1	36.8
1.5	0.5	-1	1.0	1	8.5	7.5	-1	0.03	1.5	-1	35.2
1.0	0.75	-1	1.0	-1	8.5	7.5	1	0.02	1.5	1	38.1
1.0	0.5	-1	1.0	-1	7.0	5	-1	0.02	1.0	-1	34.5

Note: Exp, Experiment; Try, Tryptone; Yea, Yeast extract; Amp, Ampicillin; Ara, L-arobinose; Ino, Inoculation ratio; N, relative quantity of N protein

Table 2. Plackett-Burman experiment results analysis

Factor	F value	P value	Importance order	Sign(p<0.1)
Tryptone	6.2	0.0677	1	*
Yeast extract	4.6	0.0984	3	*
NaCl	5.8	0.0746	2	*
pH	0.8	0.4329	5	
Ampicillin	0.2	0.7191	6	
L-arabinose	7.3e-004	0.9798	7	
Inoculation ratio	3.5	0.1366	4	

Table 3. Design and results of response surface methodology

Tryptone (%)	Yeast extract(%)	NaCl (%)	N protein (%)
0.9	1.7	2.2	31.1
0.9	0.5	1.0	28.4
1.5	1.1	2.2	32.8
0.3	0.5	1.6	31.7
0.9	0.5	2.2	32.6
0.3	1.0	1.6	35.2
1.5	1.0	1.6	34.2
0.3	1.1	2.2	35.9
1.5	0.5	1.6	37.7
0.9	1.1	1.6	44.0
0.9	1.1	1.6	44.3
1.5	1.1	1.0	26.9
0.3	1.1	1.0	33.5
0.9	1.1	1.6	44.5
0.9	1.0	1.0	35.7

Note: Y, N protein relative quantity(%); X_1, tryptone; X_2, yeast extract; X_3, NaCl

Response surface analysis chart was also generated using the software Design-expert7.0. As shown in Fig 2, apex value exists for each factor. According to a normative analysis for screening optimized regions, it was found that stable point existed for the regression model. The maximal value of Y was 44.3137, and the stable point was X_1 = -0.073, X_2 = 0.073, and X_3 = 0.052. Therefore, the optimized recipe was 0.86 % tryptone, 1.14 % yeast extract and 1.63 % NaCl.

According to optimized fermentation conditions mentioned above, five batches of fermentation for N protein expression were performed for accordance evaluation. The mean value of the relative N protein quantity in the 5 repeated experiments was 44.25%, which had only a 0.14% deviation with theoretically deduced value.

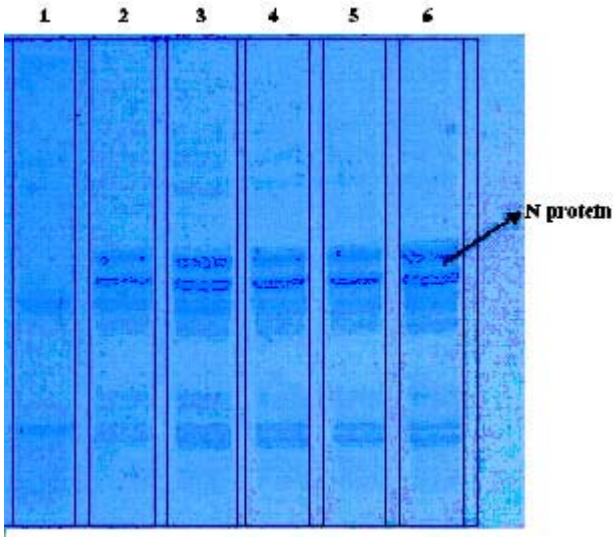
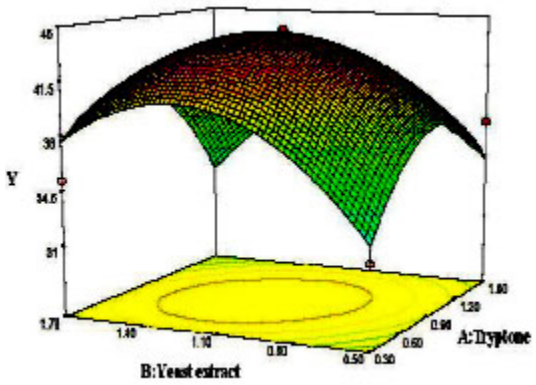
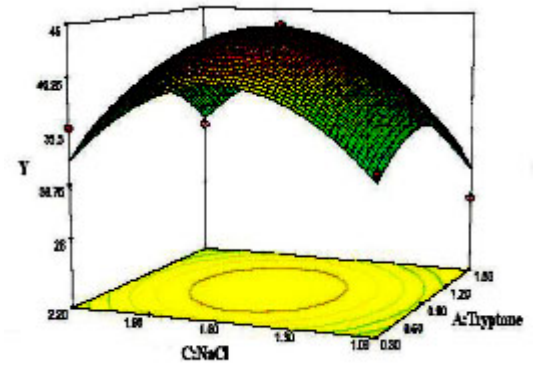


Fig. 1. The N protein expressed in the steepest ascent experiment 1, negative control (without induction); 2-6, experiment groups

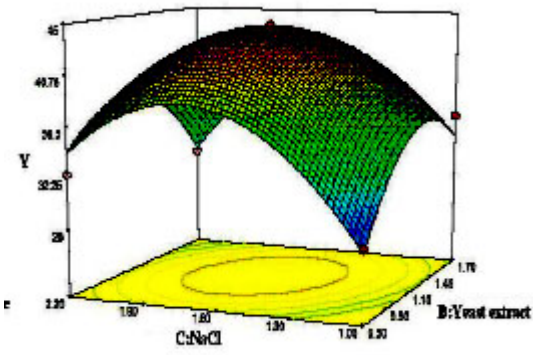
Thus, the regression equation established above showed satisfying stability.



A)



B)



C)

Fig. 2. Analysis of the response surface method results

DISCUSSION

More and more studies have pointed out the importance of N protein of coronavirus, so N protein produced *in vitro* is expected to be applied in the fields of diagnosis, vaccination and scientific research. N protein is best known as a viral structural protein, whose function is to bind to a RNA genome to form a helical ribonucleoprotein (RNP) in mature virions[15]. The N-terminal region of N protein has been indicated to be important for RNA binding in infection of bronchitis virus (IBV), severe acute respiratory syndrome coronavirus, mouse hepatitis virus and human coronavirus OC43. Furthermore, additional sites like region 2 of MHV N protein and region 3 of IBV N protein may also play a role in RNA binding[9, 16, 17]. IBV N protein is postulated to bind RNA via a lure and lock mechanism[16]. Aside from the structural functions, N protein has been implicated in several other processes. For example, SARS-CoV N protein has been shown to alter the activities of the AP-1, Akt, Erk, Jnk and p38 MAPK signaling pathways as well as induce a block in the G0/G1 phase of a cell cycle[17-19]. The association of N protein with elements of the viral genome as well as cellular RNA-binding factors strongly suggests a role in the transcription of subgenomic mRNAs. N protein has been shown to be essential for the efficient rescue of a number of infectious clones of coronavirus genomes including IBV[20]. Whilst replication and transcription can occur in the absence of N protein, the efficiency of both processes requires N protein to be provided either in *cis* or in *trans*[4, 6, 21].

In a previous study by us, N protein was successfully expressed *in vitro* using a prokaryotic system and an ELISA assay based on the produced N protein for antibody detection was evaluated[14]. The medium(2% casamino acids, 1 mM $MgCl_2$, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, and 0.2% glucose) used in that study for fermentation was prepared according to the manual from the supplier of the expression system[14]. Such components listed above like casamino acids and glucose can't be sterilized by autoclaving. It is obvious that the preparation of the above medium is time-consuming and expensive. Therefore, it is

necessary to find simpler medium to substitute it for large-scale production of N protein. In the present study, our resluts indicated that the optimized LB medium is appropriate for N protein expression, and the expression level was high.

All the factors discussed in this study were related to components of medium except one special factor, fermentation time which, in general, is relatively independent. Hence, the fermentation time for N protein expression was firstly optimized using the independent single factor experiment to facilitate the subsequent RSM.

In the RSM experiment, tryptone, yeast extract and NaCl were most important factors according to the importance evaluation experiment. Theoretically, the function of these components is mainly to supply nutritional factors for microorganisms, including carbon source, nitrogen source, osmotic pressure, and so on. It is necessary to point out that although the inductor L-arabinose seems not to be as important as the above three factors according to the PB experiment. It doesn't mean that the L-arabinose quantity is not important for N protein expression. For the expression of exogenous genes in most expression systems, inductor is always the most vital factor. The reason that the inductor didn't show importance like other factors in the PB experiment here might be due to the overdosed ranges. Because an inductor controls the promoter for gene expression directly, it is inadvisable to reduce its dose to threshold value, which tends to cause severe unstable expression of exogenous proteins.

ACKNOWLEDGEMENTS

This study was funded by Zhejiang Provincial Natural Science Foundation of China (LY12C03009, Y5110135 and Y5110130)

REFERENCES

1. Schalk, A.F. and Hawn, M.C. An apparently new respiratory disease of baby chicks. Journal of American Veterinary Medicine Association, 1931; **78**: 413-22.
2. Collisson, E.W., Williams, A., Parr, R. and Wang, L. An overview of the molecular biology of infectious bronchitis virus. Poultry Science Review, 1992; **4**: 41-55.
3. Zhou, J.Y., Zhang, D.Y., Ye, J.X. and Cheng, L.Q. Characterization of an avian infectious bronchitis virus isolated in China from chickens with nephritis. Journal of Veterinary Medicine B, *Infectious Disease and Veterinary Public Health*, 2004; **51**: 147-52.
4. Zuniga, S., Cruz, J.L., Sola, I., Mateos-Gomez, P.A., Palacio, L. and Enjuanes, L. Coronavirus nucleocapsid protein facilitates template switching and is required for efficient transcription. J. Virol., 2009; **84**(4): 2169-75.
5. Hurst, K.R., Koetzner, C.A. and Masters, P.S. Identification of in vivo-interacting domains of the murine coronavirus nucleocapsid protein. *J. Virol*., 2009; **83**(14): 7221-34.
6. Schelle, B., Karl, N., Ludewig, B., Siddell, S.G. and Thiel, V. Selective replication of coronavirus genomes that express nucleocapsid protein. *J. Virol.*, 2005; **79**(11): 6620-30.
7. Yoo, D., Song, C., Sun, Y., Du, Y., Kim, O. and Liu, H.C. Modulation of host cell responses and evasion strategies for porcine reproductive and respiratory syndrome virus. *Virus Res*., 2010; **154**(1-2): 48-60.
8. Ilkow, C.S., Weckbecker, D., Cho, W.J., Meier, S., Beatch, M.D., Goping, I.S., Herrmann J.M. and Hobman, T.C. The rubella virus capsid protein inhibits mitochondrial import. J. Virol., 2010; **84**(1): 119-30.
9. Zhou, M. and Collisson, E.W. The amino and carboxyl domains of the infectious bronchitis virus nucleocapsid protein interact with 3' genomic RNA. *Virus Res*., 2000; **67**(1): 31-9.
10. Cavanagh, D. Coronavirus IBV: further evidence that the surface projections are associated with two glycopolypeptides. *J. Gen. Virol*., 1983; **64**(8):1787–91.
11. Koch, G., Hartog, L., Kant, A. and van Roozelaar, D.J. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. *J. Gen. Virol*., 1990; **71**(9): 1929-35.
12. Boots, A.M.H., Benaissa-Trouw, B.J., Hesselink, W., Rijke, E., Schrier, C. and Hensen, E.J. Induction of anti-viral immune responses by immunization with recombinant-DNA encoded avian coronavirus nucleocapsid protein. *Vaccine*, 1992; **10**(2): 119–24.
13. Williams, A.K., Wang, L., Sneed, L.W. and Collisson, E.W. Comparative analyses of the nucleocapsid genes of several strains of infectious bronchitis virus and other coronaviruses. *Virus Research*., 1992; **25**(3): 213–22.
14. Zhang, D.Y., Zhou, J.Y., Fang, J., Hu, J.Q., Wu, J.X. and Mu, A.X. An ELISA for antibodies to infectious bronchitis virus based on nucleocapsid

protein produced in *Escherichia coli. Vet. Med.–Czech,* 2005; **50**(8): 336–44.

15. Davies, H.A., Dourmashkin, R.R. and Macnaughton, M.R. Ribonucleoprotein of avian infectious bronchitis virus. *J. Gen. Virol*., 1981; **53**(1): 67-74.
16. Spencer, K.A., Dee, M., Britton, P. and Hiscox, J.A. Role of phosphorylation clusters in the biology of the coronavirus infectious bronchitis virus nucleocapsid protein. Virology, 2008; **370**(2): 373-81.
17. Emmott, E., Munday, D., Bickerton, E., Britton, P., Rodgers, M.A., Whitehouse, A., Zhou, E.M. and Hiscox, J.A. The cellular interactome of the coronavirus infectious bronchitis virus nucleocapsid protein and functional implications for virus biology. *J Virol*., 2013; **87**(17): 9486-500
18. M. Surjit, B. Liu, V.T. Chow and S.K. Lal, The nucleocapsid protein of severe acute respiratory syndrome-coronavirus inhibits the activity of cyclin-cyclin-dependent kinase complex and blocks S phase progression in mammalian cells. *The Journal of biological chemistry,* 2006; **281**(16): 10669-81.
19. Surjit, M., Liu, B., Jameel, S., Chow, V.T. and Lal, S.K. The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. *The Biochemical journal*, 2004; **383**(1): 13-8.
20. Casais, R., Thiel, V., Siddell, S.G., Cavanagh, D. and Britton, P. Reverse genetics system for the avian coronavirus infectious bronchitis virus. *J. Virol*., 2001; **75**(24): 12359-69.
21. Almazan, F., Galan, C. and Enjuanes, L. The nucleoprotein is required for efficient coronavirus genome replication. *J. Virol*., 2004; **78**(22): 12683-8.