

Production of recombinant nonstructural 1 protein in *Escherichia coli* for early detection of Japanese encephalitis virus infection

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Summary

Japanese encephalitis is a major public health problem in South-East Asia and Western Pacific countries. The recombinant nonstructural 1 (rNS1) protein of Japanese encephalitis virus is a potential diagnostic as well as vaccine candidate. Developments of cost-effective and simple culture media as well as appropriate culture conditions are generally favourable for large-scale production of recombinant proteins. The effects of medium composition and cultivation conditions on the production of rNS1 protein were investigated in shake flask culture as well as batch cultivation of *Escherichia coli*. Further, the fed-batch process was also carried out for high cell density cultivation (HDC) of *E. coli* expressing rNS1 protein. Isopropyl- β -D-thiogalactopyranoside (IPTG) was used to induce the expression of rNS1 protein at ~ 13 g dry cell weight per litre of culture. The final dry cell weight after fed-batch cultivation was ~ 17 g l⁻¹. The Inclusion bodies were isolated and purified through affinity chromatography to give a final product yield of ~ 142 mg l⁻¹. The reactivity of purified protein was confirmed by Western blotting and Enzyme linked immunosorbent assay. These results show that rNS1 protein may be used as a diagnostic reagent or for further prophylactic studies. This approach of producing rNS1 protein in *E. coli* with high yield may also offer promising method for production of other viral recombinant proteins.

Introduction

Japanese encephalitis virus (JEV) is the most important cause of epidemic encephalitis in most Asian regions with about 35 000–50 000 cases and 10 000 deaths annually (Solomon and Vaughn, 2002). The Envelope (E) protein and nonstructural 1 (NS1) protein (Hua *et al.*, 2010) elicits neutralizing antibodies and plays an important role in inducing immunologic responses in the infected host (Xu *et al.*, 2004; Lin *et al.*, 2008; Appaiahgari *et al.*, 2009). NS1 specific antibodies have been demonstrated to provide protective immunity against dengue viruses (Amorim *et al.*, 2010) and JEV (Lin *et al.*, 1998; 2008; Xu *et al.*, 2004). In addition, the high immunogenicity of the NS1 proteins of JE, Dengue and other flaviviruses has raised considerable interest both as an antigen for diagnostic methods (Lin *et al.*, 1998; Huang *et al.*, 2001; Konishi *et al.*, 2004; Xu *et al.*, 2004; Konishi and Kitai, 2009) and as component of subunit vaccine formulations (Lin *et al.*, 1998; 2008; Xu *et al.*, 2004). For this reason, the NS1 protein is an important immunogen for a subunit vaccine and also a prospective diagnostic reagent for the improved clinical diagnosis of JEV infections.

There is no specific therapy for JE and vaccination is the only available preventive measure in addition to mosquito vector control. The internationally licensed vaccine has several limitations in terms of cost, availability and safety, apart from ethical issues (Plesner and Ronne, 1997; Appaiahgari *et al.*, 2009). Early diagnosis of disease plays an important role to forecast an early warning of epidemic and to undertake effective vector control measures. The early diagnosis of JEV infection is achieved by serodiagnosis using ELISA based on the identification of NS1 antigen (Konishi *et al.*, 2004; Konishi and Kitai, 2009) or anti-JEV IgM antibodies (Ravi *et al.*, 2006; Shrivastva *et al.*, 2008; Tripathi *et al.*, 2010). Some of ELISA tests for detection of anti-JEV IgM antibodies utilizes whole-virus antigen prepared from cell culture so it is costly and also associated with biohazard risk. Replacement of the whole-virus antigens with recombinant envelope (E) protein eliminated the biosafety risk but not the cross-reactivity problem (Ravi *et al.*, 2006). However, all these kits are expensive due to the high costs associated with antigen production, making them unaffordable for

Received 20 January, 2012; revised 24 February, 2012; accepted 27 February, 2012. *For correspondence. E-mail tripathink@gmail.com; Tel. (+91) 751 2390477; Fax (+91) 751 2341148.

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use in the developing countries where JE is mostly prevalent. Thus, there is a need to develop detection system as well as an improved JE vaccine that may be safer, cheaper and readily available. Hence, production and purification of this protein is necessary for further studies. *Escherichia coli* is the most commonly used host for heterologous protein production because it is a well-characterized organism in the genetics, physiology and cultivation condition (Lim *et al.*, 2000; Khalilzadeh *et al.*, 2008).

Protein expression level depends on cultivation conditions, such as medium composition, induction time, inducer concentration and inducer type, which can be optimized for overexpression of a recombinant protein (Manderson *et al.*, 2006; Tripathi *et al.*, 2009). Recombinant *E. coli* can be grown to high densities in complex media, semi-defined and defined media (Manderson *et al.*, 2006; Khalilzadeh *et al.*, 2008; Tripathi *et al.*, 2011). The composition of the growth media is crucial for enhancing product formation as well as reduction of inhibitory compound formation (Manderson *et al.*, 2006; Tripathi *et al.*, 2009). Furthermore, one of the most popular methods to achieve high cell density is fed-batch culture by controlling the nutrient feeding via pH, dissolved oxygen (DO) or specific growth rate (Lim *et al.*, 2000; Manderson *et al.*, 2006; Khalilzadeh *et al.*, 2008; Bhuvanesh *et al.*, 2010). Recombinant protein purification using the minimum possible steps is crucial to meet the required level of purity. Affinity chromatography is versatile and can be used for improved purification of recombinant proteins (Bhuvanesh *et al.*, 2010; Tripathi *et al.*, 2010).

There is currently a need for developing cultivation process for high yield production of recombinant NS1 protein of JEV and development of cost-effective, safe and simple diagnostics. In our earlier study, recombinant JEV nonstructural 1(rJEV NS1) protein was cloned, expressed and monoclonal antibody-based antigen capture immunoassay for detection of JEV-NS1 antigen was developed (Kumar *et al.*, 2011). The present focus of study on JE diagnosis is being targeted to develop a field based, rapid and cheap rJEV NS1 protein-based ELISA for early detection of anti-JEV IgM antibodies. In order to produce rJEV NS1 protein in *E. coli* with high yield, overexpression of this protein in a cultivation process and a purification procedure allowing efficient recovery of the protein from the resultant biomass are necessary. Thus, we have focused on the production of rJEV NS1 protein from *E. coli*, which overexpressed this protein in the form of insoluble inclusion bodies. The culture media and culture conditions optimization; batch and fed-batch cultivation were performed to maximize overall productivity of this protein. Further, characterization of this protein was carried out for its potential as a

diagnostic tool using ELISA makes its application more feasible.

Results and discussion

Expression of the recombinant JEV NS1 protein

The JEV as well as dengue virus NS1 protein is a potential candidate for the design of subunit vaccines as well as diagnostic methods. Nonetheless, generation of rNS1 protein of dengue virus from infected tissue culture insect cells is a laborious and costly, subjected to batch-to-batch variation making it difficult for routine large-scale production (Huang *et al.*, 2001). Expression of rNS1 protein of dengue virus in *E. coli* is a much cheaper and a simpler procedure (Huang *et al.*, 2001; Das *et al.*, 2009). In the present study, rJEV NS1 protein is produced in *E. coli* and used as a diagnostic reagent for detection of antibodies. The JEV NS1 coding sequence was cloned and, transformation of the *E. coli* SG13009 strain (Kumar *et al.*, 2011) was carried out. The expressed proteins, following 4 h incubation in the presence of IPTG, were monitored by SDS-PAGE. These protein bands with molecular mass of ~ 44 kDa in the insoluble protein extracts of the recombinant strain corresponded to the predicted mass of JEV NS1 protein.

The appropriate medium for rJEV NS1 protein production was analysed by using shake flask experiments. For this purpose, four different media were tested. Maximum protein yield was obtained in modified SB medium followed by super broth medium. However, defined medium produced the lowest final cell density. Chemically defined media are generally known to produce slower growth and protein titres than semi-defined or complex media (Bhuvanesh *et al.*, 2010). Thus modified SB medium was considered for further studies. The rJEV NS1 protein yield in different media are shown in Table 1.

Batch and fed-batch cultivation to produce rJEV NS1 protein

For *E. coli* or any other cultivation systems, the level of intracellular accumulation of a recombinant protein is dependent on the final cell density. Several recombinant proteins have been successfully produced in recombinant *E. coli* by fed-batch cultivation using various regimes of nutrient feeding resulting in different biomass and production yields. The development of a fed-batch process for high yield production of rJEV NS1 protein is required for further studies as a diagnostic reagent or prophylactic purpose. Batch cultivations with SB medium and modified SB medium were carried out. The dry cell weight (DCW) at the time of induction (after 5 h of cultivation) was 2.80 and 3.10 g l⁻¹ respectively. The final DCW (~ 9 h of

Table 1. Production characteristics of rJEV NS1 protein expressed in *E. coli* using different media in shake flask culture and bioreactor.

Media	Culture condition	DCW (g l ⁻¹)	rJEV NS1 Protein (mg l ⁻¹)
LB Broth	Shake flask	1.18	8.84
Super broth (SB)	Shake flask	1.77	13.02
Modified SB medium	Shake flask	2.14	16.10
Defined medium	Shake flask	1.05	6.69
Super broth (SB)	Batch cultivation	4.30	32.75
Modified SB medium	Batch cultivation	6.25	48.42
Modified SB medium	Fed-batch cultivation	17.78	142.16

growth) at harvest in all media is given in Table 1. The modified SB medium again resulted in more DCW and rJEV NS1 protein in comparison with SB medium (Table 1). This may be due to the presence of glycerol in comparison with other media. This has already been established in earlier findings where yeast extract or glycerol was used as media components (Manderson *et al.*, 2006). For further increasing the production of rJEV NS1 protein per unit volume, fed-batch cultivation using modified SB medium was carried out. The inducer (1 mM IPTG) was added to the culture at DCW of about 13.41 g l⁻¹ and allowed to grow for another 4 h before harvesting. The growth profile (OD vs. Time) during course of fed-batch cultivation is shown in Fig. 1. The control of feeding rate is maintained by keeping the DO and pH values at their set point. Yeast extract present in growth media have also been reported to enhance the specific cellular yield of the expressed protein particularly during high cell density cultivation where the demand of

nitrogenous source becomes very high following induction (Tripathi *et al.*, 2009). Glycerol is one of the commonly used carbon source. By using glycerol, high DCW and cell densities may be achieved with less frothing. The final DCW of about 17.78 g l⁻¹ was obtained at ~ 13 h of growth in fed-batch mode (Table 1). The variation of cultivation parameters with time during fed-batch process is shown in Fig. 1. The DCW and rJEV NS1 protein yield in batch and fed-batch process with different media are given in Table 1. The final DCW after fed-batch process was found to be increased more than 15 times when compared with that of shake flask culture with LB medium and about 10 times more with SB medium.

Purification and characterization of rJEV NS1 protein

Expression of heterologous protein in *E. coli* allows its rapid and economical production in large amounts. In an effort to obtain target protein in host *E. coli* strain, IBs

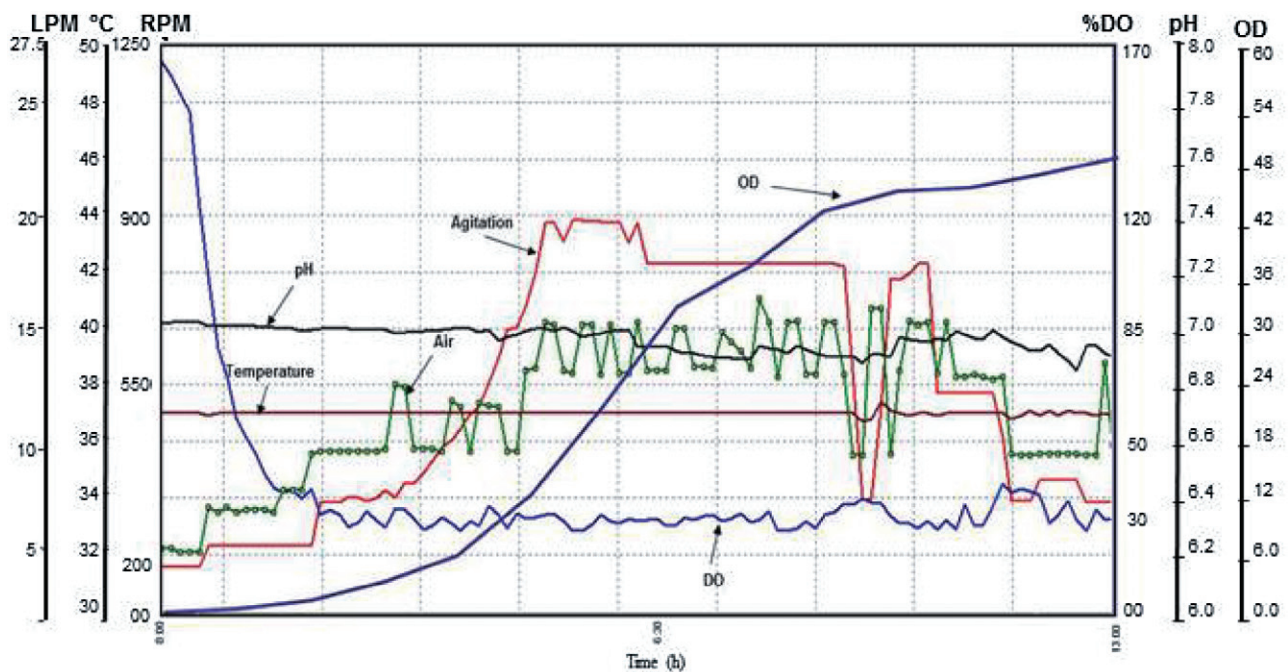


Fig. 1. Real-time profile of fed-batch cultivation for production of rJEV NS1 protein. The culture was induced with 1 mM IPTG (after ~ 9 h of cultivation) at DCW of 13.41 g l⁻¹ and cells were grown further for 4 h to attain DCW of 17.78 g l⁻¹.

formation is still considered as a convenient and effective way in recombinant protein production (Singh and Panda, 2005). After cell disruption and centrifugation analysis of the lysate confirmed the presence of the major proportion of ~ 44 kDa protein band. IBs were harvested and purified from the induced and lysed cell mass. The IBs were solubilized in buffer containing 8 M urea and purified by affinity chromatography under denaturing conditions. The purified protein was further dialysed before used for ELISA. The SDS-PAGE profile of eluted protein is shown in Fig. 2A and B. From the protein profiles of the eluted protein in SDS-PAGE (Fig. 2A and B) and densitometry analysis using Quantity One image quantification software (Bio-Rad, USA), more than 90% purity was found to be achieved. The cell pellet harvested from 50 ml of induced fed-batch culture yielded ~ 7.0 mg purified rJEV NS1 protein with ~ 92% purity. This corresponds to a recovery of ~ 50% as the crude cell lysate was estimated to contain ~ 13.9 mg of the rJEV NS1 protein and 104 mg total protein, based on densitometric analysis using Quantity One software (Bio-Rad, USA). The solubilized IBs was estimated to contain ~ 9.0 mg of the rJEV NS1 protein with ~ 64% recovery and ~ 75% purity. The final product concentration of rJEV NS1 protein following affinity chromatography was significantly higher for fed-batch cultivation as compared with batch cultivation (Table 1). Improvement in product yield about more than eight times for fed-batch cultivation as compared with shake flask culture resulted using modified SB medium (Table 1). The final rJEV NS1 protein yield after fed-batch cultivation was ~ 142.16 mg l⁻¹ (Table 1).

The purified protein was subjected to Western blot assay, to confirm its identity as rJEV NS1 protein. The purified rJEV NS1 protein was tested with mice sera raised against rJEV NS1 protein. This revealed that the rJEV NS1 protein could react with anti-JEV NS1 antibody (Fig. 2C). It is evident that this protein specifically reacted with anti-JEV antibody and thus suggests that it could be used for the purpose of diagnosis of JEV infection.

Recombinant JEV NS1 protein as a diagnostic reagent

The usefulness of purified rJEV NS1 protein for the detection of anti-JEV IgM antibodies in human sera and CSF samples was carried out by in-house developed indirect dipstick and microwell plate ELISA. A total of 50 clinical samples (30 serum and 20 CSF samples) was included. Among 30 serum samples, 16 samples were positive and 14 samples were negative and of 20 CSF samples 12 were positive and 8 samples were negative by in-house dipstick ELISA as well as in-house microwell plate ELISA. Out of 30 serum samples, 18 samples were positive and 12 samples were negative and of the 20 CSF samples 13 were positive and 7 samples were negative by JEV-CheX IgM capture ELISA. Comparison of the both in-house ELISA with JEV CheX IgM capture ELISA revealed comparable sensitivities, specificities and overall agreements (Table 2). The reaction pattern of in-house developed dipstick ELISA using rJEV NS1 protein with positive and negative serum as well as CSF samples are shown in Fig. 2D. In addition, 10 healthy serum samples as well as 5 dengue positive serum samples were also included in this

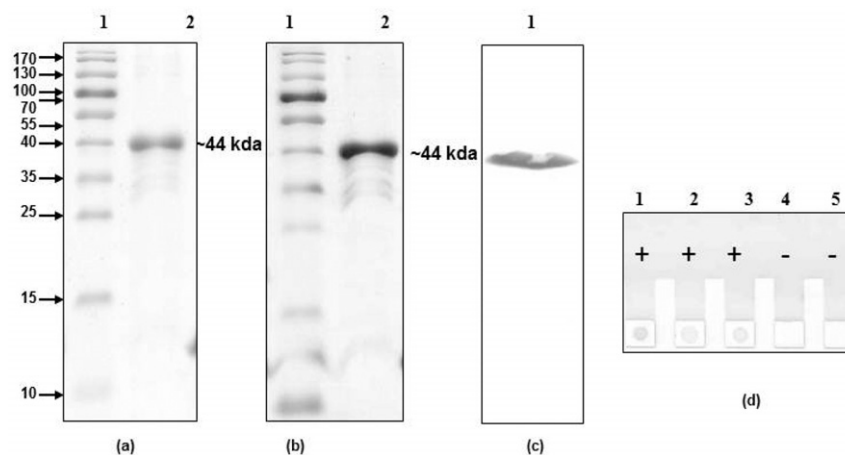


Fig. 2. A. Coomassie stained SDS-PAGE. The protein band of ~ 44.0 kDa confirmed the predicted size of the rJE NS1 protein. The protein profiles of the eluted protein in coomassie stained gel were analysed densitometrically using Quantity One image quantification software, which showed that more than 90% purity has been achieved. Lane 1, Molecular Weight Marker (kDa); lane 2, purified rJEV NS1 protein.

B. Silver stained SDS-PAGE. Lane 1, Molecular Weight Marker (kDa); lane 2, Purified rJEV NS1 protein.

C. Western blot analysis of the purified rJEV protein. The sample showing reaction with protein at desired size (~ 44 kDa) was considered positive. Lane 1, Hyper immune serum.

D. Results of Dipstick ELISA with rJEV NS1 protein. Here the sample showing a dot against a clear background were scored positive and those with no dot were scored negative. Lane 1, Positive control; lane 2, IgM positive CSF; lane 3, IgM positive serum; lane 4, IgM negative CSF; lane 5, IgM negative serum samples.

Table 2. Comparative evaluation of in-house ELISA with reference to JEV CheX IgM capture ELISA for detection of anti-JEV IgM antibodies in patient serum and CSF samples.

Type of sample	Agreement ^a	Sensitivity ^b	Specificity ^c
Serum	28/30	16/18	12/12
CSF	19/20	12/13	7/7

a. (Number of samples positive by both method + number of samples negative by both methods)/(total number of samples).

b. True positive/(true positive + false negative).

c. True negative/(true negative + false positive).

study. None of them gave positive result thereby confirming its specificity. The use of rJEV NS1 protein as an antigen in ELISA avoids costly and tedious production of viral antigen as well as the inherent biosafety issues. The diagnosis of JE has advanced considerably in recent years and routine laboratory diagnosis of JEV infection is primarily carried out by detection of anti-JEV antibody by serological methods namely MAC-ELISA and indirect IgM ELISA (Plesner and Ronne, 1997; Ravi *et al.*, 2006; Shrivastva *et al.*, 2008; Kumar *et al.*, 2011). Most of these tests are based on the capture principle and or uses native viral antigens (high production cost and produces biohazards). Therefore, there is a genuine need for a promising test system for detection of JEV infection. In the present study, the in-house indirect ELISA test has shown more than 90% agreement for detection of anti-JEV IgM antibodies in CSF as well as serum samples as compared with commercial assay. The discrepancies in correlation between commercial kit and in-house ELISA (Table 2) may be attributed to the use of crude JEV infected culture fluid as antigen in the commercial kit (Ravi *et al.*, 2006) whereas in-house ELISA uses affinity chromatography purified recombinant JEV specific nonstructural 1 protein as antigen. The advantages of this in-house ELISA test are that it is cost-effective because it uses *E. coli* expressed protein as well as faster than MAC ELISA. Further, the in-house dipstick ELISA is a qualitative test meant for field use. The negative results of rJEV NS1 protein based indirect ELISA with dengue positive human sera as well as negative serum samples established its specificity for JE diagnosis. These findings suggest that the rJEV NS1 protein based indirect ELISA is a sensitive and specific test for early detection of JEV infection.

Because the recombinant JEV NS1 protein shown high immunogenicity (Lin *et al.*, 2008), it is useful antigen for immunodiagnosis of JEV infection and also for immunoprophylaxis. For use in serological diagnosis of JEV infection as well as further studies in immunoprophylactic development, large quantity of biologically active protein was required that could be produced using bioreactor. The media optimization, batch and fed-batch cultivation strategies as mentioned in the present study demonstrated its use more appropriately. The results of in-house ELISA

using purified rJEV NS1 protein with reference to commercial assay were in good concordance. The findings of present study exhibits that the rJEV NS1 protein can be an antigen of choice for cost-effective serological diagnosis of JEV infection with acceptable specificity, sensitivity and agreement.

Conclusions

We have described here a robust and scalable cultivation method to produce recombinant JEV NS1 protein as insoluble form at 10 l cultivation scale. Usually recombinant *E. coli* is the host of choice for heterologous protein production as it is easier to grow it to higher biomass on inexpensive carbon and nitrogen sources. The composition of media affected the yield of rJEV NS1 protein production. From the economic point of view, it is necessary to increase the biomass as well as amount of protein of interest employing suitable cultivation strategies and efficient purification processes. In this study, the high cell density fed-batch cultivation was adopted to increase the protein yield. A simple, one-step purification strategy involving metal affinity chromatography was devised to produce rJEV NS1 protein with high purity. This process resulted in ~ 142 mg of purified rJEV NS1 protein per litre of culture. The purified rJEV NS1 protein thus produced has a potential application for detection of anti-JEV IgM antibody as well as further studies in vaccine development for JEV infection. This approach of creating recombinant antigens coupled to overexpression in *E. coli* and simple purification offers a promising alternative option to JE diagnosis with the potential to circumvent the drawbacks of the whole virus antigen based assays. The method described here to produce rJEV NS1 protein may also be useful in producing other viral and bacterial proteins in *E. coli* as insoluble form for production at large scale.

Experimental procedures

Bacterial strain, culture media and solution

Escherichia coli strain SG13009 was used as the host for recombinant JEV NS1 protein expression. This NS1 gene was cloned downstream of T5 promoter of *E. coli* expression vector pQE-30UA to yield plasmid pQE-JEV NS1 as previously described (Kumar *et al.*, 2011). The resultant transformants were selected on ampicillin and kanamycin plates. Stock cultures of the microorganisms were maintained in 30% glycerol (v/v) at -80°C.

Luria bertani broth (LB); Super broth (SB) (Difco, USA); defined medium (Di-sodium phosphate, 6.78 g; Sodium chloride, 0.5 g; Ammonium chloride, 1.0 g, Mono-potassium phosphate, 3.0 g, Ammonium sulphate, 1.2 g, Magnesium sulphate, 2.0 g and glycerol 20 ml per litre) and modified super broth (SB) medium (Di-potassium phosphate, 11.4 g;

Sodium chloride, 0.5 g; Ammonium chloride, 1.0 g, Mono-potassium phosphate, 2.2 g; Magnesium sulphate, 2.4 g; yeast extract, 24 g; tryptone, 12 g and glycerol 20 ml per litre). For fed-batch cultivation, modified SB medium was used as batch medium and the feed medium contained yeast extract, 300 g; glycerol, 400 ml and MgSO₄, 10 g with an antibiotic concentration of 0.5 g ampicillin and 0.25 g kanamycin per litre. For shake flasks and batch cultivations, antibiotics used were 100 µg ml⁻¹ ampicillin and 50 µg ml⁻¹ of kanamycin.

Expression of rJEV NS1 protein at shake flask culture

Trials with four different media, namely LB, SB, defined and modified SB, were done in duplicate to identify suitable growth medium for the maximal protein expression. Shake flask experiments were performed in 200 ml of respective growth medium in 1000 ml Erlenmeyer flask incubated at 37°C and 180 r.p.m. in incubator-shaker (Kuhner AG, Switzerland). The primary culture was grown by inoculating 1.5 ml of glycerol stock of *E. coli* in 50 ml LB medium with antibiotics in 250 ml Erlenmeyer flask at 37°C for 8 h at 150 r.p.m. The 2% (v/v) of primary culture was then added in 50 ml of respective medium with antibiotics and incubated at 37°C for overnight at 180 r.p.m. About 4 ml of overnight grown culture was used as the seed inoculum. After 5 h of culture growth, the cultures were induced by using 1 mM IPTG. In all the experiments, the cells were harvested after 4 h of induction by centrifugation at 4°C and 6000 r.p.m. for 10 min and the pellet was frozen at -20°C. Samples were taken for measurement of optical density at 600 nm (OD₆₀₀), DCW and rJEV NS1 protein expression.

High cell density cultivation to produce rJEV NS1 protein

Batch and fed-batch cultivations were performed to optimize the production of rJEV NS1 protein. SB and modified SB media were further used in batch cultivations to optimize the expression of rJEV NS1 protein in a bioreactor with 5 l working capacity. The optimized condition was further scaled-up to 10 l cultivation scale to produce biomass using fed-batch cultivation. Cultivations were carried out in a bioreactor (BioFlo 3000-NBSC, USA) equipped with Biocommand plus software for data acquisition and control. For inoculation in bioreactor, the 2% (v/v) of overnight grown shake flask culture in respective medium was added in 500 ml of medium and incubated at 37°C for 6–8 h at 180 r.p.m. This seed culture (5% v/v) was added aseptically to the media in the bioreactor vessel with antibiotics. The cultivations were performed with following set-up: air flow rate, 0.5–1.5 vvm; stirrer speed, 200–900 r.p.m.; pH, 6.8–7.0; temperature, 37°C. DO level was maintained at ~30% of air saturation by controlling agitation rate, air flow rate and use of pure oxygen. The pH was controlled about 6.8–7.0 by the addition of ammonia and foaming was prevented by use of antifoam agent. Sampling was performed at regular interval to measure OD₆₀₀. For the cultivations that were carried out only in batch mode, cells were induced in log phase (after ~5 h of inoculation) with 1 mM IPTG and grown further for 4 h before harvesting. For the cultivation carried out in fed-batch mode, culture was

grown in batch mode with initial volume of 8.0 l until nutrient was exhausted from medium, as indicated by the rise in DO value. Feeding of feed solutions were then started (after ~5 h of inoculation) using an inbuilt peristaltic pump in bioreactor console to achieve constant DO (DO stat feeding) as well as constant pH (pH stat feeding). The fed-batch culture was induced with 1 mM IPTG at OD₆₀₀ of 42.60 and DCW of 13.41 g l⁻¹ and cells were grown further for 4 h.

Purification of recombinant JEV NS1 protein

The bioreactor culture was harvested by centrifugation at 6000 r.p.m. for 30 min at 4°C. Purification of rJEV NS1 protein was carried out according to the method described earlier (Tripathi *et al.*, 2010). Briefly, the cell pellet was washed twice with cell wash buffer (1:20, w/v) and disrupted in lysis buffer (1:20, w/v) using sonicator (Sonics, USA) followed by centrifugation at 10 000 r.p.m. for 30 min at 4°C. The cell pellet containing the inclusion bodies (IBs) was washed with IB wash buffer followed by another wash with buffer containing 50 mM NaH₂PO₄ buffer pH 6.0, 1 mM EDTA and 200 mM NaCl and centrifuged at 10 000 r.p.m. for 40 min at 4°C. Solubilization of IBs was achieved by a mechanical homogenizer (Kinematica AG, Switzerland) for about 20 min in solubilization buffer (1:20 w/v) and centrifuged at 10 000 r.p.m. for 40 min at 4°C and filtered with 0.45 µm membrane (Millipore, USA). This filtrate containing denatured rJEV NS1 protein was loaded to the chromatography column containing Ni-sepharose slurry at a flow rate of 5 ml min⁻¹ using AKTA Explorer chromatography system (GE Healthcare, Sweden). The column was washed with equilibration buffer at pH 6.3, at a flow rate of 5 ml min⁻¹. The bound protein was eluted using with same buffer at pH 4.3. The relevant elutes were pooled together and dialysed successively against dialysis buffer (50 mM phosphate buffer, 250 mM NaCl, pH 5.8) containing progressively decreasing urea concentration (6 M, 4 M, 2 M and 1 M).

Product analysis

The culture growth was monitored by measuring OD₆₀₀ using UV-visible spectrophotometer. DCW was calculated from 2 ml of washed cell pellet after keeping it overnight at 105°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein was carried out on 12% Polyacrylamide separating gel using a Mini-Protean III electrophoresis unit (Bio-Rad, USA). Protein bands were stained with Coomassie Brilliant Blue stain and silver stain (Fermentas, USA). Total protein and pure protein was determined employing bicinchoninic acid (BCA) method (Pierce, USA). Western blot was carried out with the purified rJEV NS1 protein as method described earlier (Kumar *et al.*, 2011). Briefly, after overnight blocking in PBS with 2% BSA (Bovine Albumin Serum), the membrane was incubated with rJEV NS1 antibodies (1:100 in PBS) raised in mice for 1 h at 37°C. The membrane was further incubated with Goat anti-mouse IgG horseradish peroxidase (HRP) (Sigma, USA) conjugate for 1 h at 37°C in 1:1000 in PBS (v/v). The membrane was developed by incubation in DAB (Diaminobenzidine) and H₂O₂ substrate solution for 15 min at 37°C.

Recombinant JEV NS1 protein as a diagnostic reagent

A panel of 50 clinical samples comprising 30 serum and 20 cerebrospinal fluid (CSF) samples (Shrivastva *et al.*, 2008) was included in this study. In addition, a panel of serum samples obtained from 10 healthy persons and 5 dengue virus positive (Shrivastva *et al.*, 2008; Tripathi *et al.*, 2011) was also included in this study. In-house indirect microwell plate ELISA (quantitative) as well as indirect dipstick ELISA (qualitative) were carried out for detection of anti-JEV IgM antibodies in serum and CSF samples using purified rJEV NS1 protein.

Indirect microwell plate ELISA

For indirect microwell plate ELISA, purified rJEV NS1 protein was diluted to attain 0.5 µg 100 µl⁻¹ in buffer (Na₂CO₃ 0.1 M, NaHCO₃ 0.2 M; pH-9.6) and used for coating 96-well microtitre plates (100 µl per well) at 37°C for 1 h. The coated wells were washed once with PBS and blocked with 2% BSA in PBS overnight at 4°C. The wells were washed once again with PBS-T and then incubated for 1 h at 37°C with 100 µl serum (1:100) and CSF (1:10) samples in PBS with 0.01% Tween-20 separately. Wells were washed three times using PBS + 0.1% Tween-20 and incubated with anti-human IgM HRP (Sigma, USA) conjugate (1:2000 dilution in PBS + 2% BSA). The wells were washed once again as above and incubated with 100 µl of phosphate-citrate buffer, pH 4.5 with OPD substrate for 10 min at 37°C. Peroxidase reaction was terminated with 100 µl of 1N H₂SO₄ and the absorbance was read at 490 nm using ELISA reader.

Indirect dipstick ELISA

The in-house indirect dipstick ELISA for detection of anti-JEV IgM antibodies was also carried out. For this purpose, the purified rJEV NS1 protein was coated onto nitrocellulose (NC) combs (MDI, India) with 12 projections, at the rate of 2 µl per projection (0.5 µg) and incubated at 37°C for 1 h. The unoccupied sites were blocked with 2% (BSA) in PBS and incubated at 4°C overnight. These coated NC projections were washed with PBS-T and stored at 4°C. Serum (1:100) and CSF (1:10) samples were diluted with serum diluents (0.01% Tween-20 in PBS) and dispensed at 200 µl per well. The antigen coated combs were dipped in the wells of this microtitre plate and incubated for 1 h at 37°C. Combs were then washed with PBS-T three times, each with duration of 5 min. After washing, the combs were dipped in Goat antihuman IgM HRP conjugate (Sigma, USA), diluted (1:2000) in conjugate diluent (PBS with 2% BSA), and incubated for 1 h at 37°C. The combs were then washed as above and developed with phosphate-citrate buffer, pH 4.5 containing diaminobenzidine (Sigma, USA) and H₂O₂. Appearance of brown colour dot indicated presence of IgM antibodies. All the serum and CSF samples were also tested by JEV Chex (Ravi *et al.*, 2006) IgM capture ELISA (XCyton Diagnostics, India) as per manufacturer's protocol and the results were compared with our findings. The in-house ELISA tests were also carried out with 5 dengue positive and 10 healthy serum samples to check non-specific reactivity.

Acknowledgements

The authors are thankful to Prof. M. P. Kaushik, Director, DRDE, Gwalior for his keen interest, constant support and providing necessary facilities for this study.

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