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Abnormal growth pattern of Stbl3 *E. coli* strain harbouring classical swine fever virus rescue plasmid

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Abstract
The objective of the present study was to investigate the effect of recombinant pestiviral-based Classical Swine Fever Virus rescue plasmid (pCSFV) propagation in Stbl3 chemically competent cells. We hypothesized that transforming *E. coli* Stbl3 strain with recombinant pestiviral plasmid may somehow affect its growth pattern. To test this hypothesis, we used a novel RNA-polymerase-II driven pestiviral based pCSFV plasmid as base plasmid for incorporation of GFP reporter gene. The resulting plasmid was transformed into *E. coli* Stbl3 competent cells through the heat-shock method. Plasmid isolated from positively transformed colonies when cultivated in SB (Super Broth) showed optimum bacterial growth at 72 hours of post-cultivation, indicating the metabolic burden inside bacterial host, due to recombinant pestiviral plasmid hampering bacterial growth. Bacterial cells hosting pestiviral rescue plasmid took 12 to 96 hours to attained OD600 0.065 to 1.45. In contrast, normally bacterial cells attain ~1.6 OD600 within 16 hours of cultivation. Plasmid isolation and restriction analysis at 12-hour intervals till 96 hours showed that up to 36 hours, no plasmid could be isolated since no bacterial growth was observed; at 48 hours, significantly less concentration plasmid was obtained. About 2-3 µg/µl plasmid was isolated at 72-96 hours of cultivation. Isolated plasmid showed an expected 16.384 bp plasmid when run with an X-large DNA ladder in 0.8% agarose gel. Characterization by restriction enzyme digestion with *Stu*I *enzyme* at 72-96 hours showed 6501 bp, 5230 bp, 2730 bp, 1583 bp, and 340 bp digested products, and PCR amplified product confirmed GFP gene insertion into the pCSFV rescue plasmid showing 872 bp product in 1.5% agarose gel electrophoresis. Overall, the present study sheds light on the propagation of recombinant pestiviral-based plasmids in *E. coli* Stbl3 cells and highlights the potential benefits of using this strain to maintain such plasmids in industrial-scale bacterial cultures.

Keywords: *E. coli* Stbl3, competent cell, transformation, recombinant plasmid, *pestivirus*

Introduction
CSFV is a member of the Flaviviridae family and genus *Pestivirus* (Van Regenmortel *et al.*, 2000) [1]. It is a positive-sense, single-stranded, enveloped RNA virus and shows similarity with other *pestiviruses* like Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV). The CSFV genome comprises a single open reading frame (ORF) of approximately 12.3 kb in length, which is translated into a single polypeptide of 3900 amino acids and processed into at least twelve mature proteins. The ability to create recombinant viruses using reverse genetics techniques has accelerated research on flaviviruses. The advancement of this technology made it possible to manipulate viral RNA molecules through its complementary DNA (cDNA) copy to study the effects of these changes on viral biology at the phenotypic level. “Reverse genetics” provides a means not only to investigate the functions of various virus-encoded genes (Palese *et al.*, 1996) [2] but also to allow the use of these viruses to express heterologous genes (Sakai *et al.*, 1999) [3]. Therefore, provides a new method of generating improved vaccines and vaccine vectors. This technology was relatively easily applied to the modification of plus-stranded RNA virus genomes and to the recovery (rescue) of infectious virus from cDNA. Rice *et al.* (1989) [4] published the first reverse genetics model to analyze flaviviruses. Although it has been demonstrated that reverse genetics is an effective method for studying flavivirus, the instability of the infectious cDNA of the virus in bacteria makes it difficult to manipulate (Ward & Davidson, 2008) [5]. Several approaches have been developed to propagate flavivirus infectious cDNAs in bacteria, like use of specific cloning vectors or bacterial strains (Schoggins *et al.*, 2012) [6]. *E. coli* is considered as the standard bacterial host for molecular genetics as it has a high transformation efficiency, rapid growth

and the ability to express proteins at high levels (Cronan, J.E. 2014) [7]. However, the choice of *E. coli* strain as a host for a particular plasmid or application is crucial, as numerous other *E. coli* strains are available, each with its unique characteristics and applications. It is essential to consider some critical factors.

before choosing an appropriate *E. coli* strain, such as antibiotic resistance markers, growth rate, genotypic and phenotypic traits, plasmid compatibility, metabolic capabilities, and any potential limitations or challenges associated with a particular strain. Recombinant plasmid propagation in an inappropriate bacterial host strain may result in plasmid unclonability and instability issues. The nascent plasmid might cause growth inhibition or bacterial death due to toxic gene expression (Vidal *et al.*, 1991) [24]. Additionally, rapid plasmid loss during cell division could occur (Al-Allaf *et al.*, 2005) [25]. Repeat sequences (Bichara *et al.*, 2000) [26] and AT-rich sequences (Razin *et al.*, 2001) [27] are known to be involved in this instability in certain *E. coli* host strain. Pestiviral plasmids, which contain AT-rich stretches in the variable region of 3′ NCR (Becher *et al.*, 1998) [28], experience instability when cloned into standard *Escherichia coli* hosts like DH5α or TOP10. This instability is attributed to the deletion of regions between the LTRs, likely due to homologous recombination events (Gupta *et al.*, 1987). It is recommended to use specialized competent strain like Stbl2, Stbl3 for cloning unstable inserts such as lentiviral DNA containing direct repeats (Faisal *et al.* 2013). The present study evaluated the *E. coli* strain Stbl3 bacterial host for cloning and propagation of instability-prone pestiviral-based recombinant Classical Swine Fever Virus (CSFV) rescue plasmid.

Materials and Methods

Bacterial host strain and plasmid

The Stbl3, chemically competent *Escherichia coli* was procured from Invitrogen, Thermo Fisher Scientific, and stored at -80 °C ultra-low freezer for subsequent use and preparation of competent cells for bacterial transformation in cloning and propagation of plasmid. A novel RNA-polymerase-II driven pestiviral-based CSFV rescue plasmid (pCSFV) had been developed by Patel, C. L. *et al.* (Ann. Report, ICAR-IVRI, 2017-2018) was used as a base plasmid for our study.

Competent cell preparation and transformation

The commercially procured Stbl3 *E. coli* strain was used for the preparation of competent cell by reviving in antibiotic free SOB-agar plate. Single bacterial colony was inoculated in 5 ml antibiotic-free SOB broth and allowed to grow @ 37 °C, 180rpm for 14 to 16 hours. A 500 µl bacterial culture was reinoculated in 50 ml SOB broth without antibiotics incubated at 37 °C in a shaker incubator at 250 rpm for 2 to 3 hours until 0.25 to 0.35 OD600 value was reached. Bacterial culture was centrifuged @ 4 °C, 1500 g for 10 minutes, then washed the pellet with chilled TSS (Transformation and Storage Solution) media three times. The final pellet was resuspended in 5 ml chilled TSS media. About 100 µl cells were aliquoted in pre-chilled 2 ml microcentrifuge tubes and stored at -80° °C deep freezer for further use.

Chemical transformation by heat-shock method was performed for cloning and subcloning of plasmid DNA. Total DNA ~200 ng or 20 µl of ligation mix was diluted with 5X KCM buffer (0.5 M KCl, 150 mM CaCl2, 250 mM MgCl2) and incubated in ice for 20 minutes. Then 100 µl of ice-thawed Stbl-3 competent cell was added to the diluted plasmid DNA and incubated in ice for 20 minutes. Heat-shock treatment at 42 °C for 90 seconds was given, and immediately chilled the tube in ice for 2 minutes. Then 850 µl SOC media was added to the transformation mix to make a total volume of 1 ml and incubated in shaker incubator @ 37 °C, 250 rpm for 3 hours. The 100µl transformation mix was plated in SOB agar plate containing kanamycin (50 µg/ml) antibiotic solution to select transformed bacterial cells. The plates were incubated @ 37 °C incubator for 72-96 hours or until bacterial colonies appeared.

Culture condition for transformed bacteria and plasmid DNA isolation

The bacterial strain was grown in SB broth supplemented with 50 µg/ml of kanamycin for selection. Bacterial cultures of 5 ml were inoculated with a single bacterial colony and incubated at 37 °C, 180 rpm for 72-96 hours or until optimum growth appeared. Freshly grown bacterial culture was harvested by centrifugation at 10,000 rpm for 2 minutes at room temperature. Subsequently, plasmid DNA isolation was performed by alkaline-lysis method. At every 12 hours interval up to 96 hours, the OD600 was measured using a spectrophotometer and bacterial cultures were harvested and checked for plasmid yield and restriction analysis.

Construction of recombinant CSFV rescue plasmid

Intending to generate a recombinant CSFV rescue plasmid, we first constructed a pCMV-GFP intermediate clone by subcloning the SCP2A-5UGF-GF2T overlapping fragment into customized pCMV promoter using pVAX1 vector backbone at *NruI* and *AgeI* restriction enzyme site. Then the constructed pCMV-GFP plasmid (vector) and pCSFV plasmid (insert) were digested with *Age*I-*Bgl*II restriction enzyme to create compatible ends in both insert and vector sequence. Digested and purified products of both vector and insert was ligated @ 1:3 molar ratio using Rapid DNA ligation kit (Thermo Scientific). Ligated product was transformed into Stbl-3 competent cell following chemical transformation by heat-shock method.

Characterization of isolated plasmid by PCR and restriction enzyme digestion

Isolated pCSFV-GFP plasmid was characterized by PCR amplification of 872 bp product using forward GFP and reverse CSFV specific primers. The PCR reaction was carried out using initial denaturation at 94 °C for 5 mins, followed by 35 cycles of denaturation at 94 °C for 30 sec., annealing at 57 °C for 45 sec., extension at 72 °C for 1 min with a final extension step at 72 °C for 10 mins. PCR amplified product was confirmed in 1.5% agarose gel electrophoresis and quantified by NanoDrop® ND-1000 UV-Vis Spectrophotometer. Restriction enzyme digestion of recombinant CSFV rescue plasmid with *Stu*I enzyme in Buffer B was done at 37 °C for 3-6 hours.

Result and discussion

PCR amplification of SCP2A, 5UGF, and GF2T generated 705 bp, 542 bp, and 775 bp fragments were observed in 1.5% agarose gel electrophoresis (Fig. 1). Joining of these three fragments through overlapping PCR generated 2054 bp product (Fig.2). Customized pCMV vector generated from pVAX1 vector backbone was utilized for cloning of 2054bp

overlapping PCR fragment at *Nru*I and *Age*I restriction enzyme site to construct pCMV-GFP plasmid. The *Age*I-*Bgl*II restriction enzyme digested and purified 549 bp product of pCMV-GFP plasmid (Fig.3) was used as the vector for cloning the 10877bp insert fragment of pCSFV plasmid (Fig.4) to construct the recombinant pCSFV-GFP rescue plasmid. Stbl-3 competent cell was transformed by heat-shock method followed by ligation of 10877 bp insert and 5491bp vector fragments. At 72 hours of post-transformation, colonies appeared in the SOB-kanamycin plate and were screened by colony PCR. Positive colonies showed 872bp product in 1.5% agarose gel electrophoresis as expected (Fig. 5). Positive colonies were cultivated in eight centrifuge tubes containing of SB broth with 50 µg/ml kanamycin. At every 12-hour interval up to 96 hours, plasmids were isolated by alkaline-lysis method and subjected to restriction enzyme digestion with *Stu*I. The expected digested products observed in 1.5% agarose gel electrophoresis confirmed the intact pCSFV-GFP recombinant plasmid production in Stbl3 cells. At 12 hours, no bacterial growth was observed. After 24 hours of inoculation, bacterial growth was started with less turbidity and was optimum at 72 hours. Bacterial growth (OD600) harbouring recombinant pCSFV-GFP rescue plasmid at 12 hours' time interval is mentioned in the graph. (Fig.6). Restriction analysis and intact plasmids at different time points are given in Fig.7. From the above findings of our study, we observed that transformed bacterial colony carrying 16.384 kb recombinant pCSFV-GFP plasmid appeared at 72 hours of post- transformation in Stbl-3 competent cells and optimum growth in SB media for plasmid isolation was observed at 72-96 hours of cultivation. The highest yield of intact plasmid was observed at 72 hours and beyond 96 hours truncated plasmid observed along with the intact plasmid band in agarose gel electrophoresis.

Molecular cloning requires the assembly of recombinant DNA molecules and the transformation of the product into a host organism for replication. Chemical transformation and electroporation are the primary methods to prepare *E. coli* cells for transformation (Ren *et al*. 2019) [4]. Although electroporation induces higher transformation efficiency (Dower *et al.* 1988) [13], it is rarely utilized with standard cloning techniques since the synthesized DNA is typically purified before electroporation (Sambrook *et al.* 2000) [12]. Low cloning efficiency may also occur due to DNA loss during purification and cell injury from electroporation (Tan and Yiap 2009) [11]. The chemical transformation includes the preparation of competent cells by treating them with various divalent cations (Huang and Reusch, 1995) [10]. TSS (transformation storage solution) method of competent cell preparation is a simple one-step procedure (Datsenko and Wanner 2000) [9] followed in the present study to prepare *E. coli* strain Stbl3 competent cell. The present study was aimed to analyse recombinant pestiviral-based pCSFV rescue plasmid propagation in Stbl3 chemically competent cell. The Gram-negative bacterium

coli is one of the earliest and most frequently used bacterial host cells for plasmid DNA production due to several advantages, including its well-characterized genetics (Serres *et al*. 2001) [16]; rapid growth, high yield, high cell densities and minimum nutrient requirements (Listner *et al.* 2006) [17] availability of large number of cloning vectors (Williams *et al.* 2006) [17] and mutant host strains (Yau *et al.* 2008) [19]. However, plasmid DNA replication and maintenance within *E. coli* host cell can induce metabolic burden resulting in cell growth alterations (Sorensen and Mortensen 2005) [20]. The instability issues can arise during the initial formation of transformant colonies or throughout the subsequent expansion of the bacterial culture. In some cases, the instability could be fatal for the plasmid (Al-Allaf *et al.*, 2005) [25]. In our experiment, we observed that at higher cell density when bacterial OD reaches >1.4, growth stress triggers the recombination event in bacterial plasmid, leading to the development of truncated plasmids. Cultivation techniques and media composition are the critical parameters that can significantly affect the recombinant plasmid maintenance inside the bacterial host system (Goyal *et al.* 2009) [21]. The metabolic burden of plasmid highly influences cell growth and plasmid yield propagated inside the bacterial host (Silva *et al.* 2012) [15]. It is found that pestiviral-based recombinant plasmids are prone to instability inside bacterial propagation (Ward & Davidson, 2008) [5]. The bibliographic report suggested that an unstable lentivirus-based recombinant plasmid gained structural and maintenance stability when propagated in *E. coli* strain Stbl3, implying that in large-scale bacterial cultivations, this strain could be utilized effectively to maintain other stability-compromised plasmids (Faisal *et al.* 2013).

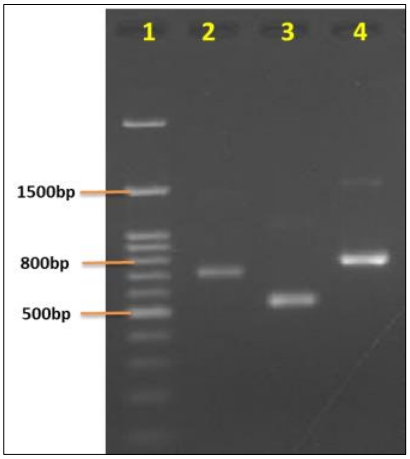

Fig 1: 1.5% agarose gel showing PCR amplified products of SCP2A, 5UGF and GF2T Lane1- 100 bp DNA ladder Lane2 – SCP2A (705 bp) Lane3 –5UGF (542 bp), Lane 4- GF2T (775 bp)

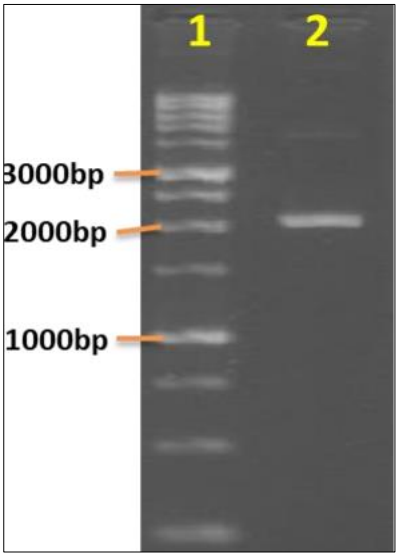

Fig 2: 1.5% agarose gel showing overlapping PCR amplified products of 2054 bp Lane1- 1kb DNA ladder Lane2 - 2054 bp product

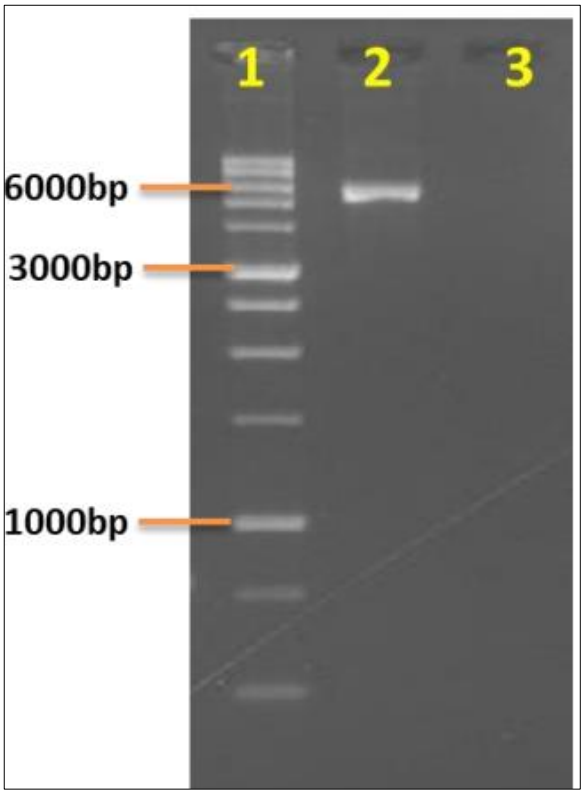

Fig 3: 1% agarose gel showing AgeI- BglII digested 5491 bp product of pVAX-GFP Lane1- 1kb DNA ladder Lane2- 5491 bp purified product

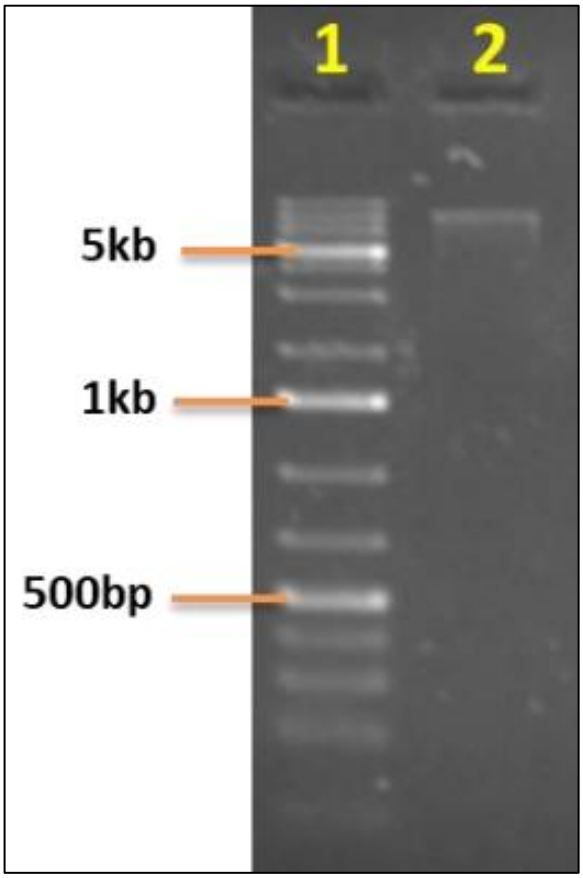

Fig 4: 0.8% gel showing AgeI- BglII digested 10877 bp product of pCSFV plasmid Lane1-1kb plus ladder, Lane2- 10877bp purified product

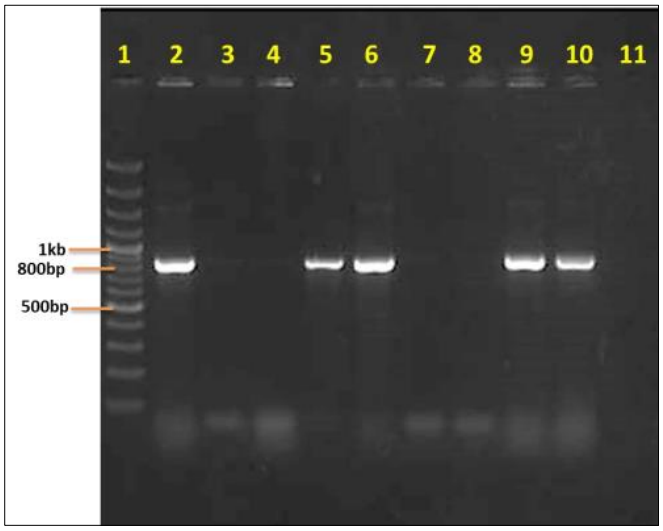

Fig 5: 1.5% gel showing 872 bp colony PCR product of pCSFV-GFP plasmid Lane1-100 bp plus ladder, Lane 2, 5, 6, 9, 10- positive colonies Lane11- Negative control

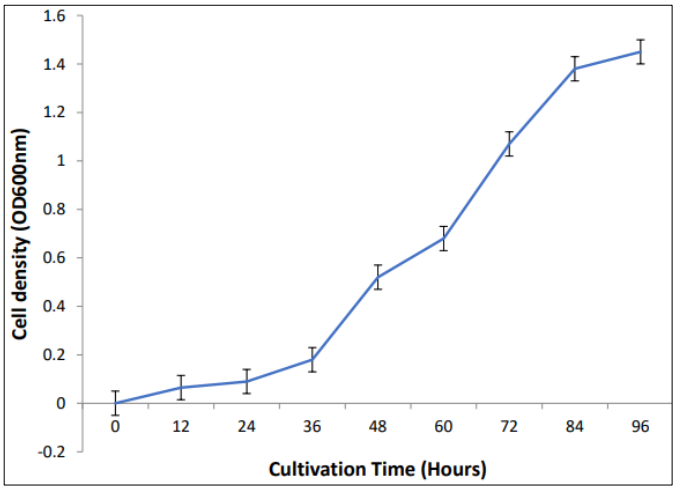

Fig 6: Line graph showing OD600 value at 12 hours' time interval for Stbl3 cells carrying recombinant pCSFV plasmid

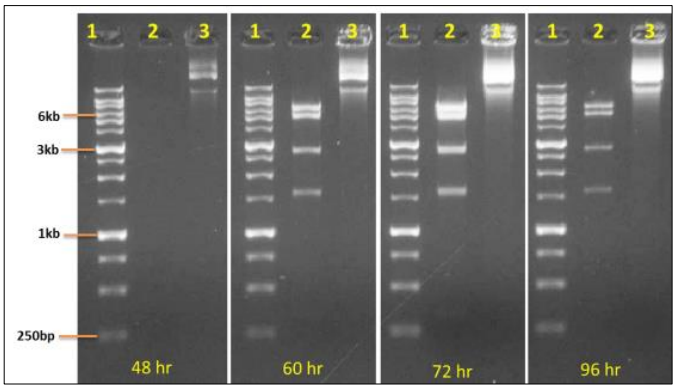

Fig 7: pCSFV-GFP plasmid and StuI digested products at different time point L- Xlarge DNA ladder, P-Plasmid, D-Digested products 6501 bp, 5230 bp, 2730 bp, 1583 bp, and 340 bp

Conclusion

In conclusion, in the present study we investigated the propagation of recombinant pestiviral- based CSFV rescue plasmid (pCSFV) in Stbl3 chemically competent cells which enables the production of intact plasmids but alters the

bacterial growth pattern, as evidenced by the bacterial growth observed during cultivation. it took significantly longer to reach a desirable OD600 compared to normal bacterial cells indicating the metabolic burden inside the bacterial host. Intact plasmid isolation and restriction analysis revealed that the optimal time to isolate the plasmid was between 72 to 96 hours of cultivation. This study provides valuable insights into the use of *E. coli* Stbl3 cells for maintaining recombinant pestiviral-based plasmids in large-scale industrial bacterial cultures, which could have significant implications for biotechnological applications.

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