



SHORT COMMUNICATION

Cloning of a Conserved Domain of ORFV- DNA polymerase in the pCDH-CMV-MCS-EF1-cGFP-T2A-Puro Lentiviral Plasmid

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ABSTRACT

Contagious ecthyma or Orf is a highly contagious; zoonotic disease and despite the application of some control strategies against it, remains prevalent worldwide. Numerous measures such as anti-viral gene therapies are frequently employed to control viral infections such as ORFV. One way to evaluate these measures is the cloning of the viral target sequence into suitable vectors for the preparation of cell lines expressing sub- genomic replicons of the virus. In this study a conserved sequence of ORFV- DNA Pol was synthesized and cloned in pCDH-CMV-MCS-EF1-cGFP-T2A-Puro lentiviral vector at the upstream of GFP gene. The validity of cloning was confirmed by restriction enzyme digestion and sequencing. Therefore, this recombinant plasmid will be available to produce lentiviral vectors carrying this gene and after infection of Eukaryotic cells with such lentiviral vectors the expression of target gene will be induced.

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INTRODUCTION

Contagious ecthyma (also called orf or sore mouth) is a viral zoonotic disease caused by a member of family Poxviridae that is prevalent all over the world. The most important symptoms in sheep are painful skin lesions often observe on the mouth and muzzle and also in udder and foot. Secondary bacterial infections can situate and sometimes extend into internal organs. Orf has been reported in people who handled infected animals or their tissues (Bergqvist *et al.*, 2017).

ORFV genome is linear double-stranded (ds) DNA with the size of of ~135 kb. It contains a highly conserved large central coding region surrounded by two equal inverted terminal repeat (ITR) regions. Essential replicative machinery, making structure of the virus and morphogenesis are encoding by central portion of the genome. Viral virulence and pathogenesis are mostly due to variable genes are posited at the terminals (Chi *et al.*, 2013).

Parapoxvirus OV-SA00 is the standard strain of ORFV includes 130 putative genes and share collinearity over 127 genes, 88 of which are conserved in all defined chordopoxviruses. 16 open reading frames (ORFs) with low likeness to other poxvirus or cellular products are distinguished in ORFV genome (Kumar *et al.*, 2015).

Recently, the application of gene therapy for the treatment of viral diseases has been increased dramatically. One way for evaluating the efficacy of gene therapies is the preparation of reporter cell lines expressing the viral genes have been as the targets of gene therapy methods. For this purpose, the first step is the cloning of viral target sequences into a suitable vector for transfection into appropriate cell line.

In the previous studies, due to the conservation of the ORFV-DNA polymerase enzyme and functional roles in virus life cycle, it has been mostly selected as a target. So, in the present study, the conserved DnaQ_like exonuclease domain of ORFV DNA pol was selected for cloning in a lentiviral plasmid (Wang *et al.*, 2014; Bergqvist *et al.*, 2017).

The lentiviral vectors have been utilized for stable and long-term expression of the desired genes in the target cells. Transfection of plasmid vectors has also been used by researchers to induce gene expression in the target cells, but the duration of gene expression is low. Lentiviral vectors (LVs) may provide highly effective gene therapy, as they can change their target cell's gene expression for up to six months. Furthermore, they encompass the efficient delivery; the ability to transduce the proliferating and resting cells and the absence of

virulence genes are some of advantages of LVs for gene therapy. In addition, LVs can transfer large nucleotide sequences (3000 bp). Moreover, due to the lower frequency of integration at or near the cellular proto-oncogenes, the possibility of mutagenesis and carcinogenesis following gene induction by lentiviral vectors is low (Henry *et al.*, 2006).

Given the reasons mentioned above, in the present study lentiviral vectors were preferred to express the target gene in the desired cells and the first step for the preparation of such vectors was the cloning of the desired gene in the transfer vector of a lentiviral packaging system.

Among the available lentiviral plasmids, pCDH-CMV-MCS-EF1-cGFP-T2A-Puro, which has ampicillin and puromycin resistance genes, was selected for ease of access and handling. Both antibiotics are easy to achieve. Furthermore, the vector has a GFP gene as a marker protein that is applicable for the evaluation of transfection efficiency. Also this plasmid passes strong RSV, CMV and EF1 promoters for expression of the desired gene.

The objective of this study was the production of a pCDH-CMV-MCS-EF1-cGFP-T2A-Puro lentiviral plasmid expressing ORFV-DNA polymerase as a transfer vector.

MATERIALS AND METHODS

Preparation of ORFV- DNA pol- DnaQ_like exonuclease: OV-SA00 - DNA polymerase sequence was obtained from NCBI database and aligned with all DNA polymerase sequences of other ORFV strains using the online software www.ebi.ac.uk/Tools/msa/clusalo to find the conserved regions. Finally, the DnaQ_like exonuclease domain was selected due to its key roles for the virus. Then the BamH I and EcoR I cleavage sites were added to the 5' and 3' ends, respectively. The His Tag sequence added to 3' end for future monitoring tests. Finally this sequence was sent to the ShineGene Molecular Biotechnology Company for synthesis.

Restriction enzyme digestion: pCDH-CMV-MCS-EF1-cGFP-T2A-Puro (in this study, briefly called pCDH) lentiviral plasmid (Addgene) and puc57 carried DnaQ_like exonuclease fragment digested by *BamHI* (Roche, Germany, Cat. No: 10220612001) and *EcoR I* (Roche, Germany, Cat. No.: 10220566001). 7.5 µl of plasmid (2 µg / µl), 1.3 µl of each enzymes (10U), 2.5 µl of 10x K buffer and nuclease-free water were added in a final volume of 25 µl. After incubation at 37°C for 16 h, 1 µl of digested plasmid was electrophoresed in a 1% agarose gel in order to confirm the restriction enzyme digestion.

Extraction of DnaQ_like exonuclease fragment from agarose gel: In order to purify the digested plasmid, the product of digestion was extracted from agarose gel using Expin Gel SV (50 prep) kit (geneall, Cat. No.: 000001422) according to manufacturer's instructions and finally kept at -20°C.

Ligation and transformation: Ligation reaction was performed with 2µl of T4 10x ligation buffer (Fermentas,

Germany, Cat. No.: B69), 2µl of 50% PEG-4000 (Fermentas, Germany), 1 µl of T4 DNA ligase (Fermentas, Germany, Cat. No.: EL0013), 150 ng of inserting DNA and nuclease-free water up to 20µl. Then ligation product was transformed into DH5α competent cells by heat shock protocol and finally was cultured in LB agar with 100 mg/ml ampicillin. Plates were placed at 37°C for 16-14 hours.

Colony PCR: After culturing the transformed bacteria, 10 individual clones of each plate were used for PCR reaction and plasmid extraction using Mini preparation of DNA method.

PCR was carried out using the general primers of pCDH-CMV-MCS-EF1-cGFP-T2A-Puro lentiviral plasmid. The sequences of primers were as follows: CMV-F: AATGGGCGGTAGGCGTGTA -3'and EF1-R: 5'-GGACTGTGGGCGATGTG -3'. The PCR thermal cycle programs were consisted of denaturation at 95°C for 5 min followed by 30 cycles at 95°C for 30 s, 55°C for 40 s and 72°C for 60 s, followed by a final extension at 72°C for 10 minutes. The positive (synthesized DnaQ_like exonuclease fragment) and negative controls (de ionized water) were employed in each test. Each PCR reaction was performed in a final volume of 25 µl containing 11 µl of deionized sterile water, 10 µl of Taq DNA Polymerase 2x Mix Red-Mgcl2 2 mM (GeneAll, Cat. no. A180301), 1 pmol of each primer and 2 µl of DNA template. After electrophoresis in 0.8% agarose, the recombinant plasmids were sent for sequencing.

RESULTS

Extraction of DnaQ_like exonuclease fragment from agarose gel and colony PCR: 1 µl of digested plasmid and 1 µl of the uncut plasmid were electrophoresed in a 1% agarose gel along with a ExcelBand™ 1 KB Plus (0.1-10 kb) DNA Ladder to observe of the extraction of fragment from digested pUC57. After culturing of transformed DH5α and positive and negative, recombinant plasmid containing and positive control colonies (containing lentiviral pCDH plasmid resistant to ampicillin that confirmed in previous experiments) were observed but in the negative control plate (only DH5α) there weren't any colonies.

After colony PCR the presence of the insert fragment was detected in transformed colonies samples with the size of 784 bp. The length of the PCR product in not cloned pCDH-CMV-MCS-EF1-cGFP-T2A-Puro lentiviral plasmid (positive control) was in the range of 213 bp. Also, no band was detected in untransformed DH5α (negative control) (Fig. 1).

Extraction of recombinant plasmid and sequencing: From two colonies that had positive colony-PCR results, a 10 mL overnight culture was prepared and then the plasmid was extracted using Mini Prep protocol. 1 µl of the extracted plasmids were electrophoresed on 1% agarose gel and the desired band was observed (Fig. 2). Sequence analysis of the recombinant plasmids revealed the presence of DnaQ_like exonuclease fragment (Fig. 3).

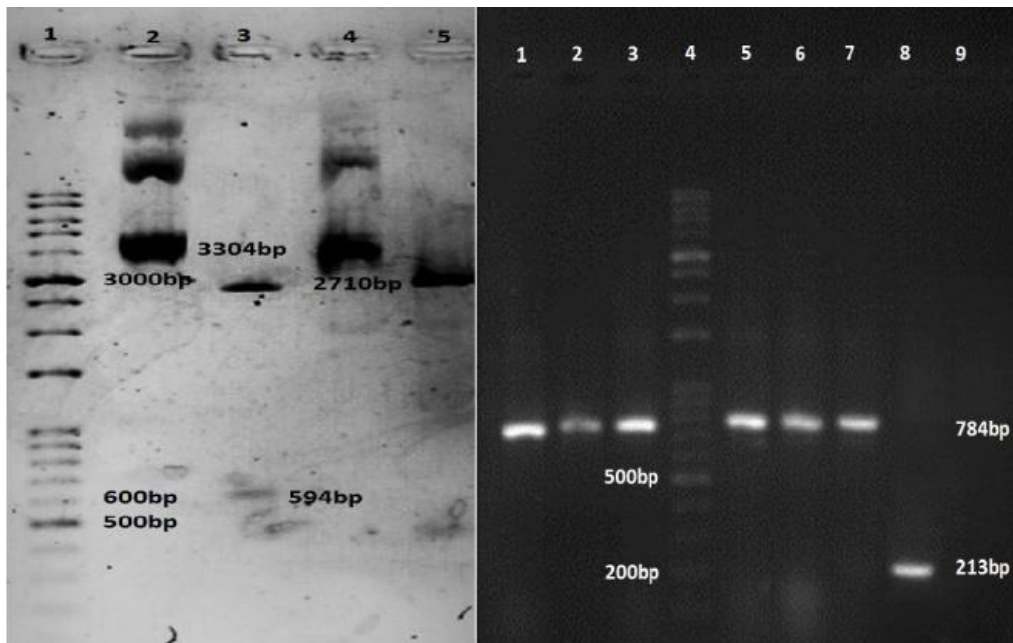


Fig. 1: Left: Extraction of digested and uncut pUC57 plasmid. Right: Colony PCR for detection of DnaQ-like exonuclease. Left, 1: Uncut pUC57 carrying insert fragment (3304 bp), 2: the band above: Digested pUC57 plasmid (2710 bp), bottom band: insert DNA (594 bp). 3: Uncut pUC57 carrying insert fragment (3304 bp), 4: Digested pUC57 plasmid (2710 bp), L: 1Kb DNA Ladder-Right, 1-3: PCR products (213 bp), 4: Ladder (100 bp), 5-7: PCR product, 8: Positive control, 9: Negative control.

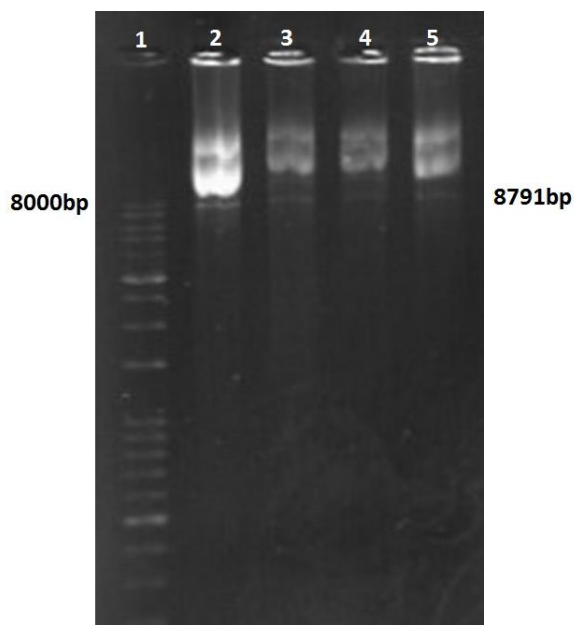


Fig. 2: Extraction of recombinant plasmid; L: 1 Kb Ladder, 1-4: recombinant pCDH plasmids in different concentrations (8791 bp).

DISCUSSION

Finding any way for ORFV treatment or control is of interest to scientists since it is a zoonotic and highly contagious agent; causes significant economic impacts on the livestock industry. The contagious nature of ORFV, its rapid transmission among susceptible hosts and long survival time in the environment are some of the difficulties to eradicate ORFV (Bergqvist *et al.*, 2017). Although some traditional therapeutic measures are used, the treatment of acute ORFV infections is usually not effective (Chi *et al.*, 2013; Said *et al.*, 2013). Given the fact that vaccination in many cases results in vaccine-

derived lesions those are virus-rich sources, the use of vaccines is limited to ORFV-affected farms or the farms with history of ORFV occurrence. Furthermore, existing vaccines don't have acceptable efficacy and vaccinated animals can be infected more than once (Kumar *et al.*, 2015). Due to the lack of specificity and side effects of existing chemical drugs, genetic science has also been involved for the treatment of contagious ecthyma. It's obvious that such genetically treatments should be evaluated. Therefore, the objective of present study was to develop an approach for evaluation of gene therapies against ORFV.

There are a number of studies in which the specific evaluation of therapeutic genetic manipulations for treatment of diseases caused by the members of poxviridae has been performed. For example, Lantermann *et al.* (2007) inhibited *vaccinia virus* gene expression by siRNAs. They used an evaluation method by producing the cell line expressing vaccinia sub genomic replicons (Lantermann *et al.*, 2007). In another work, Kilcher *et al.* (2014) applied RNAi to detect an uncoating factor of *vaccinia virus* and the specific evaluation of the interfering molecules performed using sub genomic replicon expressing cells (Kilcher *et al.*, 2014).

Wang *et al.* (2014) designed siRNAs against DNA pol gene to reduce ORFV replication. Although the reduction of viral replication was up to 89-73%, the specific evolution of target gene downregulation was not investigated (Wang *et al.*, 2014).

Except a report for the preparation of an ORFV-based viral vector, we didn't find any study in which ORFV genes were expressed using LVs. In 2003, Fischer *et al.* (2003) cloned ORFV VEGF-E and two-glycoproteins of pseudorabies in separate lentiviral plasmids, with co-transfection of them into Vero cells a new viral vector based on Parapoxviruses was created.

BLAST Results

Blast 2 sequences

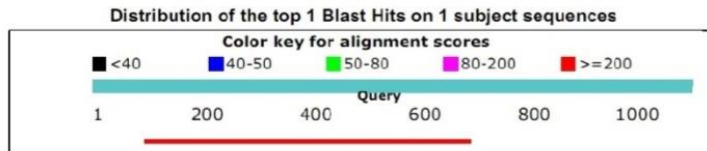
Job title: Sample_nj_2_primer_nj_F

RID PEW386MD114 (Expires on 07-14 21:06 pm)

Query ID Icl|Query_234989
 Description Sample_nj_2_primer_nj_F
 Molecule type nucleic acid
 Query Length 1074

Subject ID Icl|Query_234991
 Description None
 Molecule type nucleic acid
 Subject Length 594
 Program BLASTN 2.6.1+

Graphic Summary



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
None provided	1092	1092	55%	0.0	99%	Query_234991

Alignments

Sequence ID: Query_234991 Length: 594 Number of Matches: 1
 Range 1: 1 to 594

Score	Expect	Identities	Gaps	Strand	Frame
1092 bits(591)	0.0()	593/594(99%)	0/594(0%)	Plus/Plus	
Query 98	GAATTCATGCGACATAGAGTGCACCTTCGAGAAAAAGTTTCCTTCGTTTCAGGAACGC	157			
Subject 1	GAATTCATGCGACATAGAGTGCACCTTCGAGAAAAAGTTTCCTTCGTTTCAGGAACGC	60			
Query 158	CCTCTCCGACATCACCTTTCCTCCCTCCACACACCCGCCACACACATTCACCCCT	217			
Subject 61	CCTCTCCGACATCACCTTTCCTCCCTCCACACACCCGCCACACACATTCACCCCT	130			
Query 218	SACGAACTCCGATATGCTTCCGACCGGACCTCGAGGAGCGGGCACGGCGGAGATCCC	277			
Subject 121	SACGAACTCCGATATGCTTCCGACCGGACCTCGAGGAGCGGGCACGGCGGAGATCCC	180			
Query 270	GTTCCTGCTGAGACCCGCGAGCTGAAAGTTCGACGCGGAGGTCACCTTGTTCGCCAGGT	337			
Subject 161	GTTCCTGCTGAGACCCGCGAGCTGAAAGTTCGACGCGGAGGTCACCTTGTTCGCCAGGT	240			
Query 338	SACCGTGTTCGCGCTGGCCAAAGCGCTTCTCGAGATGCCCTGGACTTCGTGGTCACTT	397			
Subject 241	SACCGTGTTCGCGCTGGCCAAAGCGCTTCTCGAGATGCCCTGGACTTCGTGGTCACTT	450			
Query 398	CAACGGCCACAACTTCGACCTGCGGTACCTGGACTGCGCGCTGTCCCTGCTCACGGGCGA	457			
Subject 301	CAACGGCCACAACTTCGACCTGCGGTACCTGGACTGCGCGCTGTCCCTGCTCACGGGCGA	360			
Query 458	GCACATCCGCTTTCGCTTCGCGGACGGGACCGAGACATCACTTCTGGTGTACGAGCG	517			
Subject 361	GCACATCCGCTTTCGCTTCGCGGACGGGACCGAGACATCACTTCTGGTGTACGAGCG	420			
Query 518	CACGAAGTCCAGCCACAAAGGGGCTCGCGCGCTCTCGAGCACCACTTCCACATCAACAA	577			
Subject 421	CACCAAGTCCAGCCACAAAGGGGCTCGCGCGCTCTCGAGCACCACTTCCACATCAACAA	480			
Query 578	CAACAACGGGACCACTACTTTCGACCTGTACCGCTTCATCCAGCGCACCGAGAGCTGGA	637			
Subject 481	CAACAACGGGACCACTACTTTCGACCTGTACCGCTTCATCCAGCGCACCGAGAGCTGGA	540			
Query 638	CTCTACAGCTGAGAGCCATATCCACATCTCAGATGACCACTAGGATTC	591			
Subject 541	CTCTACAGCTGAGAGCCATATCCACATCTCAGATGACCACTAGGATTC	594			

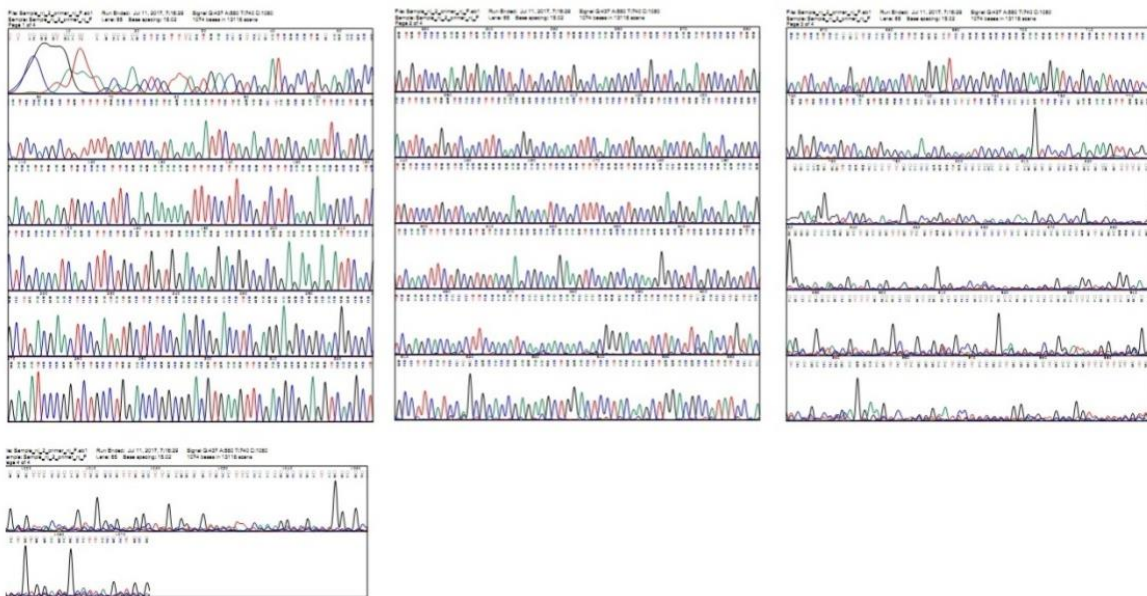


Fig. 3: Sequencing result; As shown in the picture, there was 99% similarity to the expected amplicon.

There are several reports for production of reporter cell lines expressing of sub-replicons of some other viruses. For instance, Puig Basagoiti *et al.* (2009) after cloning of target gene segments of Western Nile virus into appropriate plasmids produced a cell line expressing the WNV sub-replicons that successfully applied to evaluate the chemical inhibitors of WNV (Puig Basagoiti *et al.*, 2009).

There are reports of the preparation of lentiviral vectors expressing other viral genes, for example Wang *et al.* (2013) produced shRNA induced by lentivectors expressing GFP as a reporter protein. After cloning the target genes of Newcastle virus in the transfer plasmid and transfection, the shRNAs successfully inhibited the specific target gene expression (Wang *et al.*, 2014).

Henry *et al.* (2006) used lentiviral viruses to infect the Huh-7 cell line for expression of HCV target genes. Then changes in gene expression after RNAi induction were evaluated (Henry *et al.*, 2006).

In general, the results of the present study confirmed the proper production of recombinant DnaQ-like exonuclease pCDH. Obviously, this plasmid is ready for future procedures to produce an indicator cell line for the evaluation of therapeutic approaches against ORFV.

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Authors contribution: AM: Conceiving and design the study. BS: Conceiving and design the study. NJD:

Performing the experiments and analyzing the samples. MJHA: Performing the experiments and analyzing the samples. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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