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Construction and Rescue of a Minigenome System for Lentogenic D58 Strain of *Avian orthoavulavirus* 1 (Newcastle Disease Virus)

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ABSTRACT

Background: In reverse genetics studies, minigenomes of negative sense RNA viruses are used to model the process of virus replication and evaluate the virus rescue system in the course of construction of recombinant virus. In the current study, a minigenome (MG) rescue system for lentogenic D58 vaccine strain of *Avian orthoavulavirus* 1 (AOAV-1) (Newcastle disease virus) was developed to establish a reverse genetic system for this strain.

Methods: The minigenome was constructed by assembling the trailer and leader regions of AOAV-1 D58 strain flanking the reporter gene, *Aequorea coerulescens green fluorescence protein* (AcGFP) in a modified transcription vector. Construction of this Minigenome was done using a simple restriction free technique-Splicing by Overlap Extension PCR. The constructed minigenome was evaluated for the reporter gene expression by transfection in T7 RNA polymerase expressing BSR/T7 cell line. Further, the replication and packaging ability of the constructed minigenome were also analyzed.

Result: The reporter gene expression was confirmed in BSR/T7 cells which simulates the virus replication process and verifies the virus rescue system. In addition, this proves the applicability of restriction free cloning by Splicing by Overlap Extension (SOE) PCR for precise construction of the minigenome. The developed minigenome system can be used as a potential tool in the reverse genetic rescue of AOAV-1 D58 strain for the development of marker vaccine.

Key words: *Avian orthoavulavirus*1, AOAV-1 D58 strain, Newcastle disease, Minigenome, Restriction free cloning, SOE PCR, Reverse genetics.

INTRODUCTION

Newcastle disease (ND) is a dreadful and contagious disease affecting more than 250 species of birds worldwide (Kaleta and Baldauf, 1988). In India, ND remains a potential threat to the poultry industry causing severe economic loss. The disease caused by *Avian orthoavulavirus*1(AOAV-1) (formerly designated as Newcastle disease virus (NDV) is grouped under the genus *Orthoavulavirus* with in the new subfamily *Avulavirinae* of *Paramyxoviridae* family under the order *Mononegavirales* (ICTV, 2019). AOAV-1 strains are broadly grouped in to lentogenic (non-virulent), mesogenic (moderately virulent) and velogenic (highly virulent) on the basis of its pathogenicity for chickens (OIE 2021). The genome is non-segmented negative sense (NS) single stranded RNA genome comprising of six genes coding namely nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and the large protein (L). These genes are flanked on 3′ and 5′ ends with short non-transcribed regions called leader and trailer respectively, which are essential for replication and packaging of genome (Dortmans *et al*., 2010) To control ND, vaccines prepared from lentogenic strains preferably with ICPI less than 0.4 as recommended by OIE are commonly used as live vaccines. Mesogenic strains are used in older birds as they can cause clinical signs in birds less than 6 weeks of age. In Indian scenario, the inability of conventional vaccines to differentiate naturally infected and vaccinated birds pose great challenge in eradication of ND. Rational ND vaccine strategy based on reverse genetics allows such serological differentiation (Peeters *et al*., 2001).

However, such reverse genetic system for a lentogenic ND strain which provides a platform for genome modification and aids in the development of a candidate ND marker vaccine strain is currently not available in India. For this to be accomplished, successful reverse genetics system needs to be developed. The complex reverse genetics strategies for Negative sense RNA viruses necessitate the need for a

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verification system to evaluate the virus rescue system. This is fulfilled by minigenome (MG) system in which the role of leader and trailer sequences of AOAV-1 is utilized to construct a verification system.

MG system is an artificially constructed plasmid vector that consists of a reporter gene in antisense orientation replacing the six transcriptional units of AOAV-1 genome and flanked by the 3′ leader and 5′ trailer genomic sequences of AOAV-1 under the control of T7 promoter. The artificially constructed MG plasmid is co-transfected with the helper plasmids of AOAV-1 (NP, P and L) in T7 polymerase expression system like BSRT7/5 cell line. The expression of the reporter gene verifies the rescue system developed for AOAV-1 (Feng *et al*., 2011).

Though virus rescue system was successfully developed earlier for different strains of NDV (Peeters *et al.,* 2001; Yu *et al.,* 2012), the rescue system developed for one strain cannot be directly applied to a new AOAV-1 strain. This is because the rescue efficiency is decided by many factors, like efficient and contiguous cloning of full-length anti-genome with precise ends, following "rule of six" (Genome of *Paramyxoviridae should be divisible by six for efficient replication of virus)*, selection of suitable transcription vector, suitable source of RNA polymerase, ratio and concentration of expression plasmids used in transfection and so on.

The MG construction involves modification of plasmid vector to assemble the various DNA fragments in appropriate orientation using suitable cloning methods. The limitations of ligation dependent method like non-availability of unique restriction sites, the need for vector pretreatment restricting the choice of vector necessitates the need for alternative ligation independent cloning methods. Ligation independent cloning (LIC) method like the exonuclease mediated ligation independent cloning (LIC), recombination based cloning and PCR based cloning are considered as suitable alternatives (Celie *et al*., 2016). Among these methods, the PCR based restriction free (RF) cloning which is based on Splicing by overlap extension (SOE) PCR and employs whole plasmid amplification of insert and vector (Van Den Ent and Löwe, 2006) is used in this study as a restriction free cloning technique to perform both the cloning and vector modifications with simple reagents and without the use of commercial kits.

The lentogenic D58 vaccine strain of AOAV-1 used in this study was isolated from unvaccinated healthy chicken (Ananth *et al*., 2008) with an ICPI value of 0.14. This D58 lentogenic strain in addition to its low ICPI value has thermostable and immunogenic property. Also, the pro-inflammatory cytokine response produced by this vaccine is minimal (Ranjani *et al.,* 2019) imparting lesser post-vaccination reactions, despite effective immune response. These properties prove the potential utility of this strain to be developed as a candidate marker vaccine. In this background, genetic manipulation of this viral genome warrants development of a virus rescue system for this strain. Virus rescue efficiency could be determined by MG system (as discussed earlier). Thus, in this study, a MG system is constructed by SOE-PCR based RF cloning for AOAV-1 D58 strain with AcGFP as reporter gene. This study was proposed to verify the functionality of the virus rescue system to recover full length genome and study the applicability SOE-PCR based RF cloning as a sole cloning technique to perform both the cloning and vector modifications in the construction of MG, independent of restriction sites as a simple and reliable cloning procedure.

MATERIALS AND METHODS

Virus, plasmid and cells

The study was carried out in the year 2020 at the Department of Veterinary Microbiology, Madras Veterinary College. AOAV-1 D58 strain propagated in allantoic cavity of embryonated chicken eggs (OIE, 2022) was used for this study. The plasmid pBR_T7ter_HdvRz, a modified pBR322 vector constructed previously at the Department of Veterinary Microbiology, Madras Veterinary College was used as base vector for MG plasmid construction. BSRT7/5 cell line were maintained under G418 selective pressure in DMEM, 10% fetal bovine serum and 5% MEM amino acids. It is a T7 RNA polymerase expression system (a generous gift from Dr. Karl-Klaus Conzelmann, Max Von Pettenkoffer Institute, München, Germany).

RNA extraction and cDNA synthesis

RNA extraction from D58 AAF was done by Trizol method and cDNA was synthesized using Thermoscript cDNA synthesis kit (Invitrogen, USA) as per manufacturer's instructions.

Splicing by overlap extension PCR

Primers designing and optimization of reaction conditions for restriction free cloning were done using the online software tool available at www.rfcloning.org. These hybrid primers were used to amplify and clone each insert. The details of the primers are provided in Table 1. Splicing by overlap extension (SOE) PCR reaction was carried out to clone each insert in two steps: primary PCR and secondary PCR. Primary PCR amplified the insert with vector specific complementary strands on either side. The PCR product was gel purified using PCR clean up kit (Machery Nagel) and used as mega-primer for secondary PCR with plasmid vector as template. The secondary PCR product was treated with *DpnI* enzyme to digest the parental template and transformed in to competent *Escherichia coli* cells to propagate the plasmid with insert.

Construction of minigenome plasmids

The MG plasmid was constructed in three stages by SOE-PCR. Initially, T7 promoter sequence with additional G residues for efficient transcription initiation was cloned into pBR_T7ter_HdvRz using SOE_T7 primers. Then the trailer region (including UTR of L gene - 191 nucleotides) and leader (including UTR of NP gene-124 nucleotides) were cloned between T7 promoter and HdvRz sequences. Finally,

AcGFP in negative sense orientation was cloned between the trailer and leader sequences. The secondary SOE-PCR product at each stage was treated with *Dpn I* and transformed into competent *Escherichia coli* cells to obtain desired plasmid with insert. The final MG plasmid (pD58 mini) was sequenced to confirm the plasmid construct.

Construction of helper plasmids

The NP, P and L genes of D58 strain were cloned in pCIneo expression plasmid (Promega) to get the helper plasmids pCIneo-NP, pCIneo-P and pCIneo-L. The ORFs of NP and P genes were cloned into *EcoRI-SalI*, *XbaI-EcoRI* and *MluI-SalI* sites respectively. The details of the primers are provided in Table 1. The plasmids were verified by sequencing and their expression were confirmed by SDS-PAGE and western blotting.

Transfection experiment

The BSRT7/5 cells were transfected in triplicates with pD58_mini, pCIneo-NP, pCIneo-P and pCIneo-L at 5:2:2:1 ratio using 10 µl of Mirus LT-1 transfection reagent (Mirus, Bio LLC). The same plasmid ratio without pCIneo-L plasmid served as negative control. The transfection complex was removed after overnight incubation and the cells were maintained with fresh optiMEM and 10 % allantoic fluid as a source of external protease for cleavage of fusion protein of lentogenic strains. At 24, 48 and 72 hours post transfection; the cells were fixed with paraformaldehyde and examined for AcGFP fluorescence.

Packaging experiment

BSRT7/5 cells co-transfected with pD58 mini plasmid and helper plasmids were infected with AOAV-1 D58 strain at 24 hours post transfection to analyze the packaging ability of pD58 mini genome into viral particles. The cells were observed for fluorescence after 48 hours of infection. The supernatants were collected to infect fresh BSRT7/5 cells and again observed for fluorescence after 48 hours of infection. The supernatant collected from co-transfected cells without super-infection served as negative control.

RESULTS AND DISCUSSION

Construction of minigenome

The MG fragments (F1- Leader, F2- Trailer and F3 – EGFP) amplified using the hybrid primers (Fig 1) were used to construct the MG by SOE-PCR. The AOAV-1 trailer and Leader sequences flanking the reporter gene EGFP were precisely cloned in the modified pBR_T7p_ter_HdvRz vector as shown in Fig 2 and confirmed by sequencing. The total size of the MG plasmid (pD58 mini) was 4798 bp (Fig 3a) and the length of the MG sequence in the plasmid was 1032 bp which was confirmed by amplifying the minigenome insert (Fig 3b). The total length of the MG sequence is divisible by six and confirms to the “rule of six” an essential factor of AOAV-1 replication (Kolakofsky *et al*., 1998).

The Rf cloning used in the study to construct the MG obviates the need for vector pretreatment, sub-cloning in special proprietary vectors and employs a single enzyme for both amplification of insert and cloning compared to other LIC techniques where additional enzymes like recombinases or T4 DNA polymerases are required. Further this technique allowed precise cloning of the fragments independent of restriction sites in addition to performing vector modifications signifying that RF cloning is a simplified and a cost-effective technique that allows reliable molecular manipulations. However, the limitation with this technique is the maximum size of insert that can be cloned which was reported to be 6.7 kb (Bryksin and Matsumura, 2010) but still this technique can be potentially used in the cloning of full length AOAV-1 wherever restriction sites availability is a constraint in the genome.

Table 1: Primer sequences used in the study.

Primer	Primer ID	Sequence 5′-3′	Reference
1	SOE_T7P_F	CAACGTTGTTGCCATTGCTGCAgtaatacgactcactatagggg	Designed for
2	SOE_T7P_R	GGAGATGCCATGCCGACCCcccctatagtgagtcgtattac	this study
3	SOE_TRA_F	GCTGCAGTAATACGACTCACTATAGGGGaccaaacaaagatttggtgaatgacga	
4	SOE_TRA_R	GGAGATGCCATGCCGACCCcgaaaatcacatattaataggctcctt	
5	SOE_LEA_F	GGCCAAAAAAGGAGCCTATTAATATGTGATTTTCGcatgttggcagaaggctttc	
6	SOE_LEA_R	GGAGATGCCATGCCGACCCaccaaacagagaatccgtgag	
7	SOE_EGFP_F	GGCCAAAAAAGGAGCCTATTAATATGTGATTTTCGtcacttgtacagctcatccatg	
8	SOE_EGFP_R	ACTCGAGAAAGCCTTCTGCCAACATGgtgagcaagggcgcc	
9	pCIneo_L_F	TAATTACGCGTGCCACCATGGCGAGCTCCGGTCCTGAAAG	
10	pCIneo_L_R	GGGCGTCGACTTAAGAGTCACAGTTACTGTAATATCC	
11	pCIneo_NP_F	ACGGAATTCAGGTGTGATTCTCAAGTGCG	Li *et al.,* 2011
12	pCIneo_NP_R	GGCGTCGACTTGTCCATCAATACCCCCAGTC	
13	pCIneo_P_F	GCCGAATTCCTTAGAAAAAAATACGGGTAGAAG	
14	pCIneo_P_R	TGTTCTAGAGATCAGCCATTCAGCGCAAGGCG	

Upper case sequences represent overlap sequence with vector backbone and the underlined sequences indicate restriction enzyme sites.

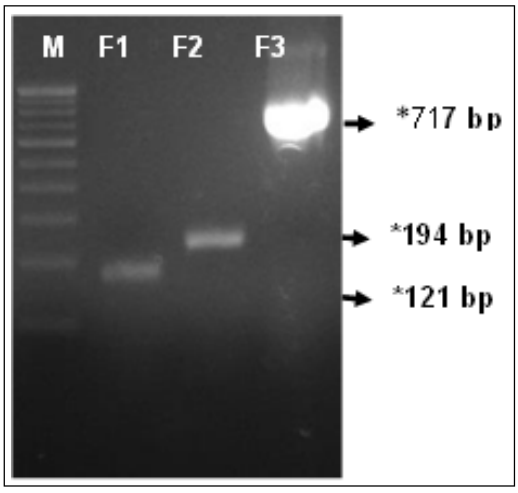

Fig 1: PCR amplification of AOAV-1 D58 minigenome fragments (F1- Leader, F2- Trailer and F3- EGFP).

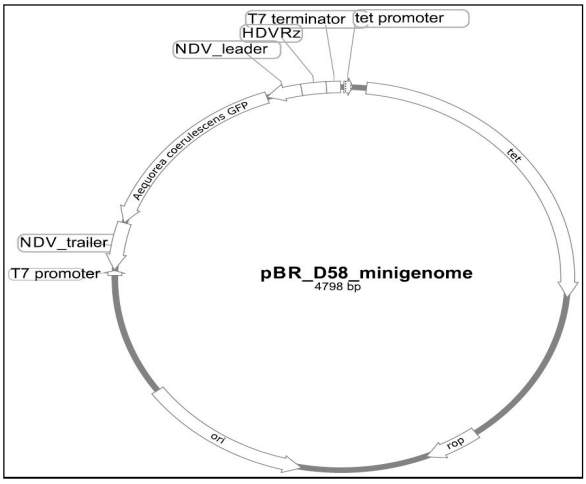

Fig 2: Plasmid map of pD58mini.

Construction and verification of expression plasmids

The amplified ORF of NP, P and L gene (Fig 4) and western blot analysis of the NP, P and L proteins showed correct amplification of each gene and their expressed proteins (Fig 5).

Verification of virus rescue system

To verify the MG construct, the BSR T7/5 cells were co-transfected with pD58 mini along with the expression plasmids, pCIneo-NP, pCIneo-P and pCIneo-L. The results in Fig 6a shows the GFP expression 48 hours post transfection compared to no GFP expression in control without pCIneo-L plasmid (Fig 6b). Initially the negative sense MG (AcGFP flanked by AOAV-1 leader and trailer) is transcribed by T7 RNA polymerase provided by BSRT7/5 cells. This negative sense RNA forms RNP complex with NP, P and L proteins provided during transfection and serves as a template for transcription of AcGFP mRNA followed by its translation into protein as evidenced by the fluorescence. The results validate the functionality of the helper plasmids and precise construction of MG as evidenced by the GFP expression.

Packaging ability of the minigenome

The co-transfected and co-infected BSR T7/5 cells showed GFP expression 72 hours after the initial transfection as shown in Fig 6c. Further, the cells infected with supernatants from co-transfected/AOAV-1 D58 infected cells also showed GFP expression (Fig 6e). No fluorescence was observed from cells inoculated with supernatants from only co-transfected cells without virus infection (Fig 6d and 6f). Further, super-infection of BSRT7/5 cells co-transfected with pD58 mini plasmid and expression plasmids after 24 hours with lentogenic D58 virus revealed fluorescence after 48 hours of super-infection. The supernatant obtained from this was used to infect fresh BSRT7/5 cells, which also showed

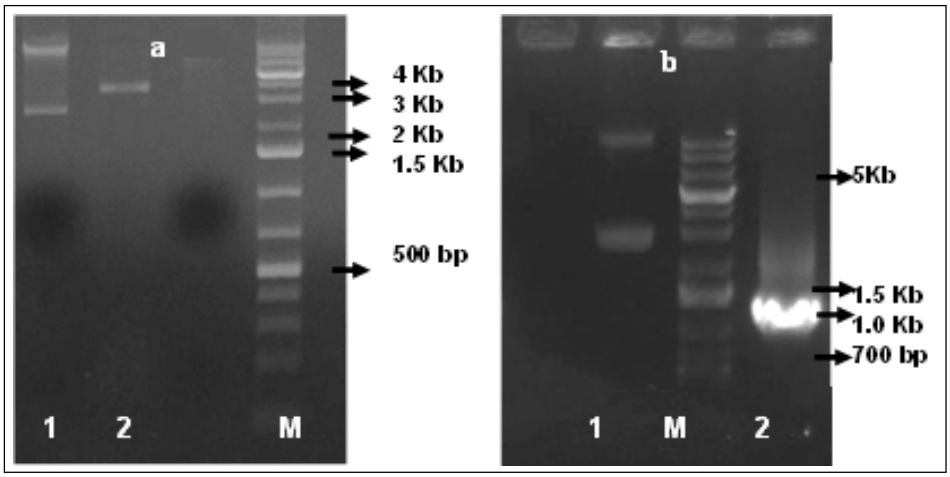

Fig 3: Identification of pD58-mini. a. restriction enzyme digestion. Lane 1: pD58 mini (uncut), Lane 2: pD58 mini linearized and Lane M: 1 Kb plus molecular size marker. b. PCR analysis of pD58-mini. Lane 1: pD58 mini (uncut), Lane M: 1 Kb plus molecular size marker and Lane 2: amplification of the complete minigenome insert to confirm the size of the insert 1032 bp.

fluorescence after 48 hours. This shows that virus envelope proteins provided by AOAV-1 co-infection was utilized by MG transcripts to be packed into viral particles which had the capacity to infect fresh cells. This confirms the packaging ability and replication ability of the MG constructed for lentogenic D58 strain of AOAV-1.

Validated and functional minigenomes of negative sense RNA virus (NSRV) have provided valuable insights to model the process of virus replication, study the role of trans-acting proteins, quantify virus and screen for antivirals (Nelson *et al*., 2017). A useful extension of the minigenome is the generation of infectious virus from cDNA copy of the genome using reverse genetics. Reverse genetics provides scope for development of new generation of AOAV-1 vaccines like marker vaccines (Peeters *et al*., 2001), vector vaccines and attenuated vaccines.

In Indian scenario, despite the control and preventive strategies followed, ND still remains endemic as there is no marker vaccine available to eradicate the disease. In the context of improved vaccines for ND eradication, an ideal vaccine strain should be of lentogenic pathotype as recommended by OIE and possess marker potential. Further in Indian conditions, in remote places where the cold chain maintenance of vaccine is challenging, thermostability of the vaccine is also essential. This necessitates the need to develop a marker vaccine with a lentogenic and thermostable vaccine strain. The lentogenic D58 vaccine strain used in this study has thermostable property. The ICPI of this strain is 0.14, which fulfills the OIE recommendations for an AOAV-1 vaccine strain. Currently as there is no reverse genetic system for a lentogenic AOAV-1 strain in India, the developed minigenome system have potential application in rescue of this D58 strain with DIVA properties. Further the minigenome can be extended to study the effect of mutations or genome modification incorporated in the D58 genome for imparting DIVA properties.

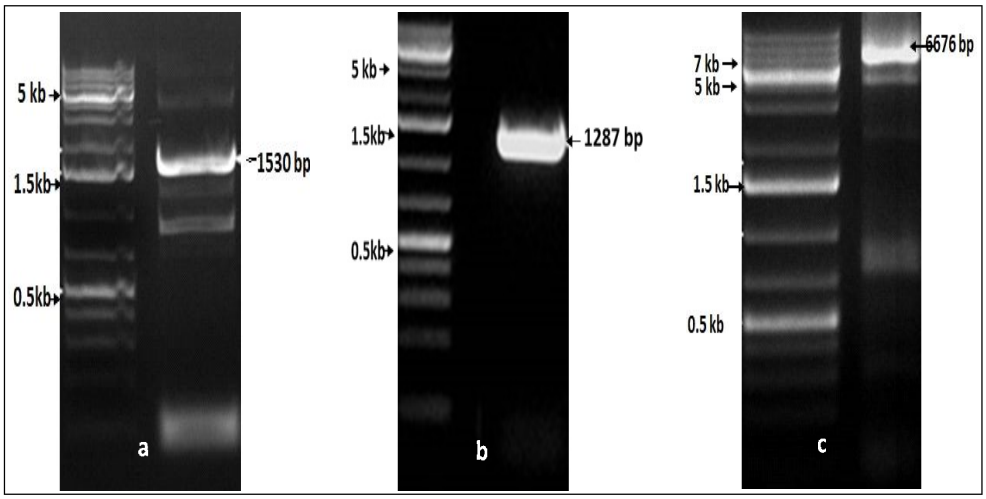

Fig 4: Amplification of coding sequence of NP, P and L genes of AOAV-1 D58 strain.
Amplification of a.Nucleoprotein gene (1530 bp). b.Phosphoprotein gene (1287 bp) and c.Large polymerase protein gene (6676bp).

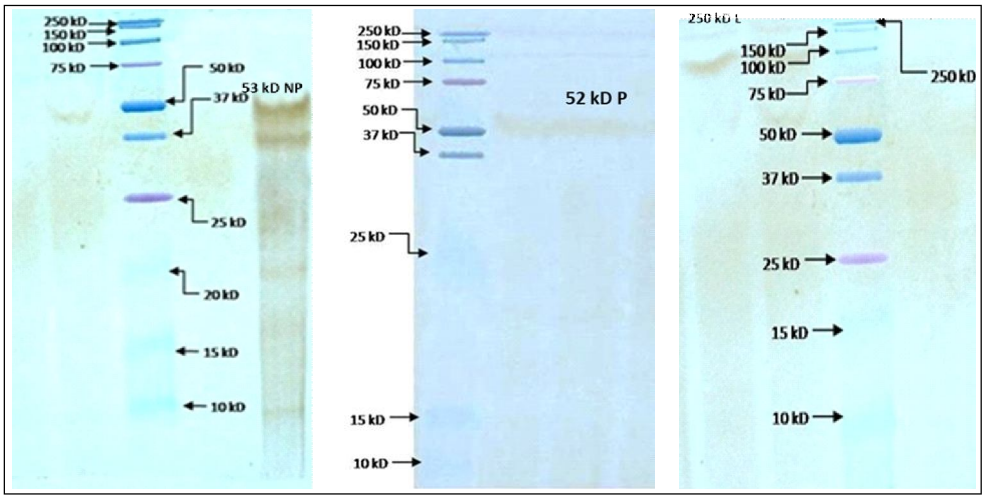

Fig 5: Western blot analysis of recombinant NP, P and L proteins of AOAV-1 D58 strain expressed in *Escherichia coli* using chicken NDV hyperimmune serum.

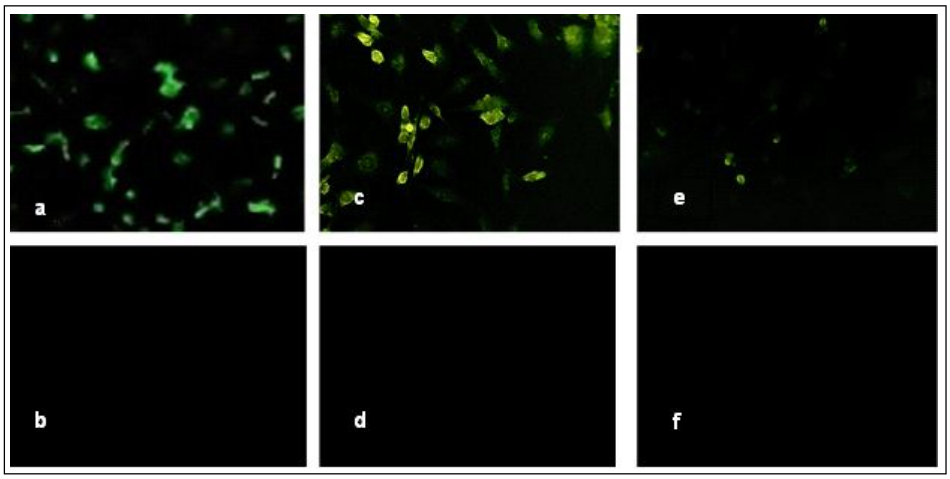

Fig 6: Transfection experiments for rescue of AOAV-1 D58 minigenome in BSR/T7 cells. Fluorescence microscopy of BSR/T7 cells (100X) a. Positive fluorescence observed 48 hours post transfection; b. No fluorescence in negative control cells co-transfected without pCIneoL; c. Transfection experiments to demonstrate the packaging ability of minigenome. BSR/T7 cells co-transfected with plasmids and co-infected with AOAV-1 D58 strain at 24 hours post infection. e. the supernatants collected from the cells were used to infect fresh BSR/T7 cells and GFP fluorescence was observed 48 hours post infection; d and f No fluorescence in negative control cells.

CONCLUSION

In the present study, the MG for lentogenic D58 strain of AOAV-1 was constructed using simplified and cost-effective SOE-PCR based RF cloning technique. This would serve as a more suitable cloning platform for reverse genetic studies which allows flexibility to optimize varied molecular manipulations. Further, the MG rescue system developed in this study should be suitable for the recovery of full length lentogenic AOAV-1 D58 strain for the purpose of developing marker vaccine that would pave way towards eradication of AOAV-1.

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Statement of conflict of interest

All authors declare no conflicts of interest.

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