Reverse Genetics Mediated Recovery of Infectious Murine Norovirus

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Abstract

Human noroviruses are responsible for most cases of human gastroenteritis (GE) worldwide and are recurrent problem in environments where close person-to-person contact cannot be avoided. During the last few years an increase in the incidence of outbreaks in hospitals has been reported, causing significant disruptions to their operational capacity as well as large economic losses. The identification of new antiviral approaches has been limited due to the inability of human noroviruses to complete a productive infection in cell culture. The recent isolation of a murine norovirus (MNV), closely related to human norovirus but which can be propagated in cells has opened new avenues for the investigation of these pathogens.

MNV replication results in the synthesis of new positive sense genomic and subgenomic RNA molecules, the latter of which corresponds to the last third of the viral genome (Figure 1). MNV contains four different open reading frames (ORFs), of which ORF1 occupies most of the genome and encodes seven non-structural proteins (NS1-7) released from a polyprotein precursor. ORF2 and ORF3 are contained within the subgenomic RNA region and encode the capsid proteins (VP1 and VP2, respectively) (Figure 1). Recently, we have identified that additional ORF4 overlapping ORF2 but in a different reading frame is functional and encodes for a mitochondrial localised virulence factor (VF1).

Replication for positive sense RNA viruses, including noroviruses, takes place in the cytoplasm resulting in the synthesis of new uncapped RNA genomes. To promote viral translation, viruses exploit different strategies aimed at recruiting the cellular protein synthesis machinery. Interestingly, norovirus translation is driven by the multifunctional viral protein-primer VPg covalently linked to the 5' end of both genomic and subgenomic RNAs. This sophisticated mechanism of translation is likely to be a major factor in the limited efficiency of viral recovery by conventional reverse genetics approaches.

Here we report two different strategies based on the generation of murine norovirus-1 (referred to as MNV herewith) transcripts capped at the 5' end. One of the methods involves both in vitro synthesis and capping of viral RNA, whereas the second approach entails the transcription of MNV cDNA in cells expressing T7 RNA polymerase. The availability of these reverse genetics systems for the study of MNV and a small animal model has provided an unprecedented ability to dissect the role of viral sequences in replication and pathogenesis.

1. RNA Transcription and Capping for The Recovery of Infectious MNV

This protocol is designed to allow the efficient recovery of infectious MNV from cDNA via in vitro transcription and its subsequent in vitro capping (section 1.1). The resulting capped transcripts are then transfected into cells to recover infectious MNV (sections 1.2 and 1.3). This approach provides the most sensitive method for the recovery of MNV with typical yields in excess of 10^5 infectious units per 35 mm (in diameter)-dish of cells for MNV. The protocol is detailed below:

1.1 Synthesis of infectious capped MNV transcripts:

1. Digest the plasmid containing the wild type MNV cDNA (pT7:MNV 3’Rz) with NheI to obtain linear DNA. NheI recognises a unique restriction site after the 3’ end polyA tail of MNV genome (Figure 2). Linearised plasmids are typically purified with using silica columns (e.g. GFX PCR DNA Gel Band Purification Kit from GE Healthcare) and eluted in H2O.

2. In vitro transcribe the linearised vector using T7 RNA polymerase as previously described. Many commercial kits are available for this purpose and provide a reproducible method of large amounts of RNA synthesis such as MEGAScript (Life Technologies) and RibomAX (Promega). Transcription reactions are typically DNase digested prior to further analysis; however in many instances this is not required as lithium chloride purifications as described below do not precipitate DNA efficiently.
1. One day before transfection, seed Raw264.7 cells at an estimated 50% confluency. Typically two T75 flasks of cells are required for 3
subsequent re-infection. As a result, typical yields will approach in excess of 10^7 infectious units after 48 hours.

2. The day of transfection, scrape the cell monolayers into Dulbecco’s modified Eagle medium (DMEM) containing 10% foetal calf serum (FCS), ensuring you generate a single cell suspension by repeated pipetting.

3. Determine the concentration of viable cells in a haemocytometer using trypan blue exclusion to label the non-viable cells.

4. Pellet the cells at 1,200 X g for 5 min and resuspend them in DMEM containing 10% FCS at a final concentration of 8 x 10^5 cells/ml.

5. Just prior to transfection, aliquot 1 ml of cells per transfection and pellet them at 1,200 X g for 2 min. Remove the media and wash the cells in 500 μl of PBS (without Mg^2+ /Ca^2+). Spin down the cells again at 1,200 X g for 2 min. Note it is advisable to keep cells in DMEM for as long as possible as storage in PBS for long periods of time may compromise the cell viability and transfection rate.

6. Remove PBS from tubes and add 130 μl of resuspension solution (Neon transfection system kit, Invitrogen) to a final concentration of 6 x 10^5 cells/ml. Care should be taken to resuspend the cells avoiding the formation of bubbles which will cause sparking during transfection and compromise cell survival.

7. Add the appropriate amount of capped MNV transcript to the cells (Figure 2), generally 1.3 μg of capped RNA is added to 130 μl of resuspended cells and mixed gently. Then, collect 100 μl of the mixture in the 100 μl Neon transfection tip. Special care should be taken to ensure that no bubbles are formed in the electroporation cuvette (Neon transfection system kit 100 μl tip) as this will cause failure of the experiment.

8. Electroporate the cells using a single pulse at 1,700V for 25 msec, ensuring the absence of sparks during pulsing which will indicate the presence of bubbles in the sample. In the case sparking should occur, discard the sample and repeat the transfection. Release the cells into an Eppendorf tube containing 1 ml of antibiotic free DMEM containing 10% FCS. Note each tip can be reused up to three times with the same RNA sample if larger numbers of transfected cells are required.

9. Thereafter, distribute the cells from the tube into independent wells containing an appropriate amount of prewarmed antibiotic free DMEM containing 10% FCS. As a general guidance, 150 μl of the cell suspension generated during step 1.2.8 are sufficient for a single well of a 24-
6. The resulting transfection mix should be then added drop-wise to the cell monolayer and the plate should be gently shaken in perpendicular

4. To proceed with the transfection of the infectious plasmid, firstly remove the media from the infected cells, wash with 2 ml of media (10% FCS

2. Remove cell culture media and add 700 μl of FPV-T7 to each well (Figure 3). A multiplicity of infection (MOI) of ~0.5 PFU per cell, based on

1. Trypsinise a monolayer of BHK-21 cells (or BSR-T7 cells), seed 7.5 x 10^5 cells into a 35 mm diameter dish in antibiotic-free growth media and incubate the cells at 37 °C with 10% CO_2 overnight. Double the amount of cells in each plate if the transfections are planned for the same day as the seeding, and allow cells to adhere to the plate for 2-3 hours at 37 °C with 10% CO_2. Note that other cells that are suitable to this approach include human 293T cells, human hepatocellular carcinoma HuH7 cells and African green monkey Cos7 cells.

3. Prepare a mixture of 1-2 μg of capped MNV transcript into 100 μl of Opti-MEM (Invitrogen) and mix it with 4 μl of Lipofectamine 2000 previously mixed in 100 μl of Opti-MEM. Mix the sample thoroughly by pipetting it up and down 15 times. Leave the mixture at room temperature for 20 minutes.

6. Incubate the cells at 37 °C and 10% CO_2 for 24 to 72 hours. Afterwards, release infectious virions from cells by freeze and thawing and determine virus titre by plaque assay or TCID50. Typical yields of around 1 x 10^5 TCID50/ml are reached.

10. Incubate the cells at 37 °C and 10% CO_2 for 24 to 72 hours. Then, release infectious virions from cells by one (or more) freeze and thaw cycles and determine virus titre in the sample using either plaque assay or TCID50. Note that lysates should be clarified by centrifugation for 1-2 minutes at maximum speed or by their filtering through a 0.22 μm pore filter prior to titration. Typically, MNV reaches titres of around 1 x 10^7 TCID50/ml at 24 hours post-transfection and up to 1 x 10^8 at 72 hours post-transfection.

11. The presence and stability of mutations introduced in pT7-MNV 3'Rz are typically determined by sequencing the rescued viruses after 2 to 5 additional passages in Raw264.7 cells.

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directions.

2. Direct Recovery of Infectious MNV from cDNA in Cells Expressing T7 RNA Polymerase

This protocol is designed to allow the recovery of MNV in cells by the transfection of an infectious plasmid harbouring the full genomic cDNA sequence by a T7 polymerase expressed in the cells. Different cell lines can be used to recover infectious MNV by this approach although we typically obtain the highest yields with BHK-21 and BSR-T7 cells 15. We typically use BSR-T7 cells since they grow faster than the parental BHK clone line. Cells are infected with fowlpox (FPV) encoding for T7 RNA polymerase (FPV-T7) 18 which functions as a helper virus to drive expression of the viral RNA and subsequent recovery of infectious virus (Figure 4). Although BSR-T7 cells constitutively express T7 RNA polymerase, this expression is not sufficient to rescue infectious MNV after transfection of pT7-MNV 3'Rz in the absence of helper FPV-T7. Whilst the typical yields from this system are at least 10-fold lower than those described above, this approach does provide a rapid method of screening mutants to allow the identification of debilitating mutations. Typically this method is used first to assess the viability of a cDNA construct. Should a construct either fail to produce infectious virus or appear to yield virus at lower levels than that of the wild type infectious clone, then the RNA based approach described above is undertaken.

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3. Prepare a mixture of 1-2 μg of capped MNV transcript into 100 μl of Opti-MEM (Invitrogen) and mix it with 4 μl of Lipofectamine 2000 previously mixed in 100 μl of Opti-MEM. Mix the sample thoroughly by pipetting it up and down 15 times. Leave the mixture at room temperature for 20 minutes.

4. Add the transfection complexes containing capped MNV transcripts in a drop-wise fashion to the cell monolayer and gently shake the plate in perpendicular directions.

5. Incubate the cells at 37 °C and 10% CO_2 for 24 to 72 hours. Afterwards, release infectious virions from cells by freeze and thawing and determine virus titre by plaque assay or TCID50. Typical yields of around 1 x 10^5 TCID50/ml are reached.

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6. The resulting transfection mix should be then added drop-wise to the cell monolayer and the plate should be gently shaken in perpendicular
directions.

7. Incubate the FPV-T7 infected, MNV plasmid-transfected cells at 37 ºC and 10% CO_2 for 24 to 72 hours. Cells transfected with infectious plasmid pT7-MNV 3'Rz normally render titres from 1 x 10^5 to 5 x 10^3 TCID50/ml.
3. Representative Results

Both reverse genetics approaches are highly efficient for the recovery of infectious MNV in cell culture as shown in Figure 5. Infectious MNV with titres exceeding $10^5$ TCID50/ml are recovered at 24 hours after transfection of capped MNV RNA into Raw264.7 cells. Similarly, the transfection of infectious plasmid pT7:MNV 3’Rz into BSR-T7 cells previously infected with helper FPV expressing T7 (FPV-T7) led to viral titres largely exceeding $10^4$ TCID50/ml (Figure 5). These viral titre values obtained with synthetic RNA and DNA molecules are similar to those obtained in transfections involving natural VPg-linked RNA isolated from infectious virions into the same cells (Figure 5). These results highlight the high efficiency of the reverse genetics approaches described here to recover genetically defined MNV variants in cell culture.

Figure 1. Illustration of MNV genome and plasmid for the recovery of infectious virus. A, Schematic representation of MNV genome organisation. Each protein coding region is illustrated as a single white box. ORF1 is translated into 7 different non-structural proteins (NS1/2 to NS7) that are released from precursor polyprotein after self-proteolytic processing. ORF 2 encodes the major capsid protein VP1, ORF 3 encodes the minor capsid protein VP2, and ORF4 overlapping with ORF2 coding region encodes virulence factor VF1. Genomic and subgenomic RNAs contain a polyA tail at their 3’ ends of variable length. B, Plasmid containing MNV cDNA used in our reverse genetic approaches (pT7:MNV 3’Rz). MNV cDNA is fused to a polyA tail of 26 residues in its 3’ end. The MNV cDNA sequence is located immediately downstream of a truncated T7 promoter sequence, to allow T7-driven transcription, and upstream of a unique Nhel site and a DNA sequence coding for a self-cleaving ribozyme after it. These sequences are instrumental for ensuring RNA transcription termination right after the genomic polyA tail present at the 3’ end.

Figure 2. Overview of the protocol for the recovery of infectious MNV from RNA transcribed and capped in vitro. The plasmid pT7:MNV 3’Rz is linearised immediately downstream of the MNV genomic sequence using Nhel restriction enzyme (step 1). After DNA purification, MNV RNA transcripts are generated in vitro by using T7 RNA polymerase (step 2). Transcription products usually run with an apparent mobility of 2.5-3Kb on a non-denaturing 1% agarose gel (step 3, Figure 3). The template DNA is eliminated using a commercial RNase-free DNAse. RNA
is then purified from free nucleotides by LiCl precipitation (step 4). The purified RNA product may then be *in vitro* capped after being previously heated at 65 °C to unfold secondary RNA structures (steps 5-6). After purification by LiCl precipitation, RNA is transfected into either Raw264.7 cells (Neon transfection system, Invitrogen) or BSR-T7 cells (Lipofectamine 2000, Invitrogen) (steps 7-8). Once inside the cell, capped RNA transcripts will be translated into viral proteins which would catalyse viral transcripts replication into new MNV RNA molecules containing a proper VPg molecule at their 5’ end. Successive cycles of replication accompanied of viral translation would generate large numbers of viral genomes which will be encapsidated to generate infectious virions. To facilitate virus release from cells, one or several cycles of freeze and thaw are performed (step 9). Viral yields can be then determined by TCID50 or plaque assay procedures.

Figure 3. Analysis of MNV RNA transcripts integrity along the protocol. A, Integrity of MNV RNA synthesised *in vitro*. The plasmid pT7:MNV 3’Rz is firstly linearised using *Nhe*Ⅰ restriction enzyme. After DNA purification, MNV RNA transcripts are generated *in vitro* by using T7 RNA polymerase (lane 2). RNA is then purified from free nucleotides by LiCl precipitation (lane 3). Transcription products are run on a non-denaturing 1% agarose gel in parallel to 1-Kb DNA ladder (New England Biolabs, lane 1). Relative mobility of viral transcripts under non-denaturing conditions is similar to a dsDNA product of 2.5-3 Kb. B, Integrity of MNV RNA transcripts after capping. MNV transcripts purified previously by LiCl precipitation (lane 2) are subjected to enzymatic capping (lane 3) and purification by LiCl precipitation (lane 4). C, Analysis in an Agilent RNA 6000 Nano chip of MNV transcripts (second lane) and capped MNV transcripts (third lane) which have been previously precipitated in LiCl. A ssRNA ladder is run in parallel.
Figure 4. Overview of the protocol for the recovery of infectious MNV from cDNA. Initially, BSR-T7 (or BHK) cells are infected with a recombinant fowlpox virus (FPV) expressing the bacteriophage T7 RNA polymerase (FPV-T7) (step 1). The infected cells are incubated for 2 hours before further treatment to allow the expression of FPV proteins which includes the recombinant T7 RNA polymerase (step 2). Afterwards, pT7:MNV 3'Rz is transfected into the cells by using Lipofectamine 2000 (Invitrogen) (step 3). Once inside the cell, pT7:MNV 3'Rz is recognised by T7 RNA polymerase which synthesises MNV RNA transcripts (step 4). The presence of a self-cleaving δ-Ribozyme sequence at the 3'end of the genome guarantees the transcript 3' terminus is located just after the polyA tail (step 5). Some viral transcripts are intracellularly capped by an FPV capping enzyme (step 6). The resulting MNV capped transcripts will be translated to generate MNV proteins which would catalyse MNV transcripts replication. Newly synthesised MNV RNA molecules containing a proper VPg molecule at their 5' end would undergo successive cycles of replication accompanied by viral translation which may finally result in the generation of infectious encapsidated virus. To facilitate virus release from cells, one or several cycles of freeze and thaw are performed (step 7). Viral yields can be then determined by TCID50 or plaque assay procedures.

Figure 5. Representative results of virus titres obtained from different reverse genetics approaches described in the text. Grey bars represent the virus titres obtained at 24 hours after Neon-transfection of 2 x 10^6 Raw264.7 cells, or after lipo-transfection of 2 x 10^6 BSR-T7 cells with in vitro transcribed and capped MNV RNA. White bars represent the virus titre typically obtained after lipofection of pT7:MNV 3'Rz (MNV cDNA) into 2 x 10^6 BSR-T7 cells previously infected for 2 hours with fowlpox virus expressing recombinant T7 polymerase (FPV-T7). As a positive control for the transfection into Raw and BSR-T7 cells, we typically use 2 μg of RNA extracted from cells infected with MNV which contain high levels of VPg-linked MNV RNA. Negative controls have been carried out with either MNV RNA or pT7:MNV 3'Rz encoding a frameshift mutation (F/S) which abrogates replication, resulting in no detectable virus (data not shown).

Discussion

Here we have illustrated two different reverse genetic approaches that allow the recovery of infectious MNV in cell culture. Both approaches effectively bypass the absolute requirement for the covalently linkage of VPg to the 5' end of the viral RNA genome via the generation of capped MNV transcripts that are then recognised by the cellular ribosomes. In vitro transcription followed by enzymatically capping is more efficient in the recovery of infectious MNV than transcription of infectious plasmids in cells expressing T7 RNA polymerase, in which the transcripts may be capped by the FPV capping enzymes. Virus titres recovered with these reverse genetics systems are similar to these obtained by transfection of viral VPg-linked RNA purified from infected cell cultures (Figure 5). The transfection of capped MNV RNA into permissive Raw264.7 cells renders a virus titre only 1-log lower than experiments involving the transfection of total RNA from infected cells containing viral VPg-linked RNA (Figure 5). This fact encourages further investigations to determine whether the addition of a VPg molecule to the 5' end of transcripts generated by these systems could result in increased virus yields which may reveal functional aspects underlying MNV infectivity in cells associated to
VPg. Nevertheless, we regard this reverse genetics system as a highly efficient one comparable to other RNA viruses reverse genetics systems currently used in which the in vitro transcribed RNA allows the recovery of titres only 10-100 lower than in real infections with virions 18,20.

Overall, the current methodologies constitute a significant step forward in the field of norovirus molecular biology and provide us with the tools to investigate the functional roles of proteins and conserved RNA motifs in norovirus genomes. These approaches have already been combined with current mouse model available and have shown that MNV recovered from infectious cDNA is able to cause lethal infection of >80% STAT1-/- mice in less than 10 days 19-21. Making use of this system we have recovered viable murine norovirus mutants of the capsid protein and of a polypyrimidine tract involved in the binding of different host factors (PTB and PCBP) that display a somewhat attenuated phenotypes in vivo 21,22. In addition, we have also recently demonstrated that viruses lacking the ability to express the VF1 protein from ORF4 efficiently replicate in cell culture but again have reduced virulence in mice with respect to WT MNV 23. These studies encourage us to design attenuated versions of human noroviruses based on MNV studies which could be investigated as potential vaccine candidates.

Disclosures

We have nothing to disclose.

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