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Short communication

# Infectivity of a recombinant murine norovirus (RecMNV) in Balb/cByJ mice

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ABSTRACT

The infectivity of a recombinant murine norovirus (RecMNV) strain, previously isolated following *in vitro* coinfections, was evaluated *in vivo* in comparison with its parental strains (MNV-1-CW1 and WU20) in Balb/cByJ mice *via* measurement of weight loss and estimation of viral loads in faeces, tissues and organs 48 and 72 h post-infection. The presence of infectious virus in all analysed tissues and organs suggests that, similarly to its parental viruses, RecMNV can disseminate beyond organs associated with the digestive tract. Our results also suggest that recombination occurring *in vitro* between two homologous murine norovirus strains can give rise to a chimeric strain which, despite slight differences, shows similar biological properties to its parental strains. This study provides the first report on *in vivo* replication of a recombinant norovirus strain isolated following *in vitro* coinfection. These results have great significance for norovirus genetic evolution and future vaccine development.



## 1. Introduction

Human noroviruses (HuNoVs) are recognised as the major worldwide cause of acute, nonbacterial, epidemic and sporadic gastroenteritis. NoVs belong to the genus *Norovirus* within the *Caliciviridae* family and are small, non-enveloped viruses with a single stranded positive sense RNA genome (Thorne and Goodfellow, 2014). They are divided into at least six genogroups (GG), with all strains detected in the murine species clustering within GGV (Martella et al., 2011; Scipioni et al., 2008). The first murine norovirus (MuNoV) strain, MNV-1, was isolated in 2003 and is described as sporadic lethal pathogen in severely immunocompromised mice, associated with signs of encephalitis, meningitis, hepatitis and pneumonia (Karst et al., 2003). The MuNoV genome is divided into four open reading frames (ORF), wherein ORF1 encodes a polyprotein which is co-and post-translationally cleaved into non-structural proteins, ORF2 the major capsid protein VP1, ORF3 the minor capsid protein VP2, and ORF4 a virulence factor VF1 (Thorne and Goodfellow, 2014).

The MuNoV, which was found to propagate and form plaques in an immortalised mouse macrophagic cell line (RAW 264.7) (Wobus et al., 2004), remains the sole NoV which can be efficiently and easily replicated *in vitro*. Moreover, MuNoVs have been shown to be infectious when inoculated by oral or intranasal route and to naturally spread between immunocompetent mice (Hsu et al., 2005; Mumphrey et al., 2007). Thus the murine model offers the advantage of being an affordable model for *in vivo* experimentation and is considered a good surrogate for HuNoV studies (Katayama et al., 2014; Wobus et al., 2006).

Recombination events have frequently been detected *in silico* for NoVs and usually occur at the ORF1/2 overlap (Bull et al., 2007).

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Some of the latest epidemic HuNoVs have been characterised as recombinants (Eden et al., 2013). Recombination clearly plays a crucial role for genetic evolution of HuNoVs and could present a hurdle to future vaccine development. Previously, we successfully recovered a viable recombinant MuNoV (RecMNV) amongst the progeny viruses of two co-infecting MuNoV isolates (MNV-1CW1 and WU20) in RAW 264.7 cells (Mathijs et al., 2010). "RecMNV" showed smaller plaque size and reduced replication kinetics compared with the parental strains. Here we evaluate the infectivity of RecMNV *in vivo* in comparison with the parental viruses.

## 2. Materials and methods

### 2.1. Viruses and cells

MuNoV isolates MNV-1 (CW1) (passage 5), WU20 (passage 5) (Thackray et al., 2007) and RecMNV (passage 1) (Mathijs et al., 2010) were propagated in RAW 264.7 cells (ATCC TIB-71) grown in Dulbecco's modified Eagle's medium (Invitrogen), complemented (DMEMc) with 10% heat inactivated FCS (BioWhittaker), 2% penicillin (5000 U/ml) and streptomycin (5000 mg/ml) (PS; Invitrogen) and 1% HEPES buffer (1 M; Invitrogen). Concentrated and purified virus stocks were produced as previously described (Mathijs et al., 2010).

### 2.2. In vivo *experiments*

Twenty-four seven-weeks-old female Balb/cByJ wild-type SPF mice (Charles River, Belgium), obtained from a commercial colony tested negative for MuNoV infection by ELISA, were orally inoculated with $5 \times 10^6$ plaque forming units (pfu) of each MuNoV virus parental strain in 100 μl of phosphate buffered saline (PBS) using a feeding needle. In parallel, mock-infected mice were inoculated with 100 μl of non-infected cell-culture supernatant. Animals were handled following the procedures approved by the University of Liège animal ethical committee with the amendment n° 960 and were housed as groups in microisolator cages with unlimited access to a commercial diet and water.

The mice were separated into four distinct groups of six mice. All manipulations were carried out in the following cage order: i) mock; ii) RecMNV, iii) WU20 and iv) MNV-1, separated by thorough disinfection of material and equipment and systematic changes of gloves to avoid cross-contamination. Faeces and blood were sampled before virus inoculation. Body weights were monitored and both blood samples on EDTA and faecal samples were collected at 0, 24, 48 and 72 h post infection (hpi). Three mice per group were euthanized at 48 and 72 hpi. From each animal, spleen, mesenteric lymph nodes (MLN), small intestine, and left lung were removed and stored at −80 °C. Blood and organs were homogenized (10%, [weight/volume]) in complemented Dulbecco's modified Eagle's medium (DMEM) prior to further analysis. The experiment was repeated a second time, with the same conditions excepting the gavage, which was performed without anaesthesia. The infectious doses were confirmed to be similar *via* back titration, using a plaque assay.

### 2.3. Virus detection by RT-qPCR

#### 2.3.1. RNA extraction

Viral RNA was extracted from 100 μl cell culture, blood or organ supernatants with the TRI Reagent® Solution (Applied Biosystems) according to manufacturer's instructions. RNA pellets were resuspended in 30 μl of nuclease-free water.

#### 2.3.2. cDNA synthesis

First-stranded cDNA was generated with an iScript cDNA Synthesis kit (Bio-Rad) according to manufacturer's recommendations.

#### 2.3.3. Quantitative real-time PCR (qPCR)

qPCRs were performed using an iCycler Thermal Cycler (Bio-rad) with a multiplex qPCR discriminating between MNV-1 and WU20 as previously described (Mathijs et al., 2010). Two μl of cDNA (from samples and standards for MNV-1 and WU20) were added to a 20 μl reaction volume containing 10 μl of iQ Supermix (Bio-Rad). Amplification cycles were performed as follows: 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 40 s at 60 °C. Viral genome copy numbers were calculated by interpolation from a standard curve. The limit of detection (LOD) was estimated at 60 cDNA copies per 10 mg of tissue or faeces (100 μl of supernatant).

#### 2.3.4. Preparation of DNA constructs as standards

A 469-bp PCR product for MNV-1 and WU20, including nucleotide positions 6828–7260 in the MNV- 1 genome (GenBank Accession Number AY228235), were amplified as previously described (Mathijs et al., 2010). Both products were cloned into a pGEM-T Easy cloning vector (Promega) and transformed into *E. coli* DH5α competent cells. Circular plasmids were purified according to the manufacturer's instructions with the Plasmid Midi Kit (Qiagen). Plasmids were further digested by the PstI restriction enzyme (New England Biolabs) for linearisation before purification with the QIAquick Gel Extraction Kit (Qiagen). Numbers of DNA copies were calculated based on the concentration measured by spectrophotometry (Nanodrop, Isogen) and were serially diluted to defined concentrations for the elaboration of standard curves for MNV-1 and WU20 for quantification.

### 2.4. Virus titration and isolation by plaque assay

Virus titres were determined by plaque assay as described by Hyde et al. (2009). To avoid cell cytotoxicity, tissue samples were additionally diluted 5 times and blood samples 15 times. The LOD was 1–3 pfu/100 μl supernatant. Viruses were isolated from plaques as previously described (Mathijs et al., 2010). Isolated viruses were further characterized by sequencing 300–600 bp stretches in 5 regions of each MuNoV genome as described previously (Mathijs et al., 2010). Sequence analyses and alignments were carried out in the BioEdit Sequence Editor software version 7.0.9.0 (Hall, 1999).

### 2.5. Statistical analysis

The body weight of the mice was standardised by index. Index 100 was attributed to the body weight of each animal measured before infection. The average values of each parameter were compared between RecMNV and parental (MNV-1 and WU20) viruses by means of Welch test with a Bonferroni correction *via* simultaneous comparisons (Rec MNV *versus* MNV-1 and RecMNV *versus* WU20). Statistical significance was defined as $P < 0.05/k$, with k being the number of comparisons made. GraphPad Prism was used for graphical representations. In all graphs vertical bars indicate standard deviations from the mean values. For viral burdens in organ tissues, horizontal bars represent the mean values. Asterisks represent P values inferior to $P < 0.017$.

## 3. Results and discussion

In the present study, we evaluated the infectivity of a recombinant MuNoV (RecMNV) *in vivo* by comparing weight loss, viral loads in faeces, blood and various organs of RecMNV-infected