3.2.2 *In vivo* characterisation of an *in vitro* generated recombinant murine norovirus

In preparation

Elisabeth Mathijs[1], Fabiana Dal Pozzo[1], Claude Saegerman[2], Etienne Thiry[1]

[1]Veterinary Virology and Animal Viral Diseases, [2]Epidemiology and risk analysis applied to veterinary sciences, Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium

INTRODUCTION

Human noroviruses (HuNoVs) are the major cause of acute, nonbacterial, both epidemic and sporadic gastroenteritis worldwide. NoV belongs to the *Caliciviridae* family along with genus *Lagovirus*, *Nebovirus*, *Vesivirus* and *Sapovirus*. Caliciviruses are small, unenveloped viruses containing a single stranded positive sense RNA genome (Green, 2007). The genome is divided into three open reading frames (ORF) encoding respectively a polyprotein for non structural proteins, the major capsid protein (VP1) and the minor capsid protein (VP2). The ORF1-encoded polyprotein is further cleaved by the viral proteinase into six mature products with the gene order N-term, NTPase, p18–20/22, genome-linked virus protein (VPg), proteinase and polymerase (Sosnovtsev *et al.*, 2006). NoVs are divided into 5 genogroups (GG) based on their genomic composition. HuNoVs belong to genogroups I, II and IV whereas GGIII and GGV enclose bovine NVs (BoNoVs) and murine NVs (MNVs) respectively.

The Murine Norovirus 1 (MNV-1) was described as sporadic lethal pathogen in severely immunocompromised mice associated with signs of encephalitis, meningitis, hepatitis and pneumonia (Karst *et al.*, 2003). The MNV was found to propagate and form plaques in RAW 264.7 cells, an immortalised mouse macrophage and dendritic cell line and constitutes up to date the only efficient cell culture system for NoVs (Wobus *et al.*, 2004). Moreover, MNV-1 is infectious when inoculated by per oral or intranasal route and spreads naturally 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 the MNV is nowadays considered as the most suitable surrogate for NoV studies in the absence of an efficient replication alternative for HuNoVs (Wobus *et al.*, 2006). Most experimental data rely on MNV-1 studies that have been conducted under disperse conditions for: i) the inoculation dose, ii) the immunological status of infected mice, iii) organs analysed, iv) duration of the experiments and v) the detection methods rending the comparison of the results extremely delicate.

MNV was shown to be one of the most prevalent pathogen in research mice and its clearance from laboratory animal facilities is fastidious (Kitajima *et al.*, 2009; Mahler and Kohl, 2009). Viruses isolated from different breeding colonies showed the existence of a variety of MNV strains (Hsu *et al.*, 2006; Thackray *et al.*, 2007) and intertypic recombination events were suggested by phylogenetic analysis (Muller *et al.*, 2007; Thackray *et al.*, 2007). These studies

suggested the recombination site to be located within 100 nucleotides of the ORF1-ORF2 overlap or within the ORF2. Previously, we successfully recovered a viable recombinant MNV (Rec MNV) among the progeny viruses from two co-infecting wild-type MNV isolates (MNV-1 and WU20) in RAW cells (Mathijs *et al.*, 2010). Its chimeric genome showed maximum homology with WU20 in ORF1 and MNV-1 in ORF2/3 with the crossover point located within a highly conserved stretch at the ORF1-ORF2 junction. *In vitro* characterisation of Rec MNV in comparison with the parental viruses suggested that recombination could generate viruses with distinct biological properties from the parental viruses.

In the present study, Rec MNV virulence was evaluated *in vivo* in comparison with the parental MNV-1 and WU20 viruses by comparing viral loads in various tissues at 48 h and 72 h post-infection (hpi). Virus titres in faeces, blood and organ tissues were determined in parallel either by plaque assay or RT-qPCR. Moreover, the undertaken study constitutes a first report on virulence and tissue distribution of the previously reported WU20 wild-type MNV virus.

MATERIAL AND METHODS

Viruses and cells. MNV isolates MNV-1.CW1, WU20 (Thackray et al., 2007) and Rec MNV (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 ml21) and streptomycin (5000 mg ml21) (PS; Invitrogen) and 1% HEPES buffer (1 M; Invitrogen). Virus stocks were produced as previously described (Mathijs *et al.*, 2010). All three viruses were plaque purified at least 3 times prior use in experiments.

***In vivo* experiments.** Seven-week old female Balb/cByJ wild-type mice (n = 24) (Charles River, Belgium) were orally inoculated, by using a feeding needle, with 5.10^6 plaque forming units (pfu) of MNV virus in 100 µl of phosphate buffered saline (PBS). Mock infected mice were inoculated with 100 µl of non-infected cell culture supernatant. Mice were treated according handling procedures approved by the ethical committee of the University of Liège and housed per group in microisolator cages with unlimited access to a commercial diet and water. Four separate groups of 6 mice were MNV-1, WU20, Rec MNV and mock-infected respectively. All manipulations were realised in the following order: i) mock; ii) Rec MNV, iii) WU20 and iv) MNV-1 separated by thorough disinfection measures of material and equipments in order to avoid cross-contaminations. Faeces and blood were taken before virus inoculation. Body weight was monitored at 48 and 72 hpi. Faecal samples were collected daily until 72 hpi. Three mice per group were sacrificed at times 48 and 72 hpi. From each animal, blood was collected on EDTA and spleen, mesenteric lymph nodes (MLN), small intestine, left lung were removed and stored at -80°C. Blood and organs were homogenised (10 %, [weight/volume]) in DMEMc prior virus detection and quantification by plaque assay and RT-qPCR. For clarity, the experimental design is schematised in Figure 14.

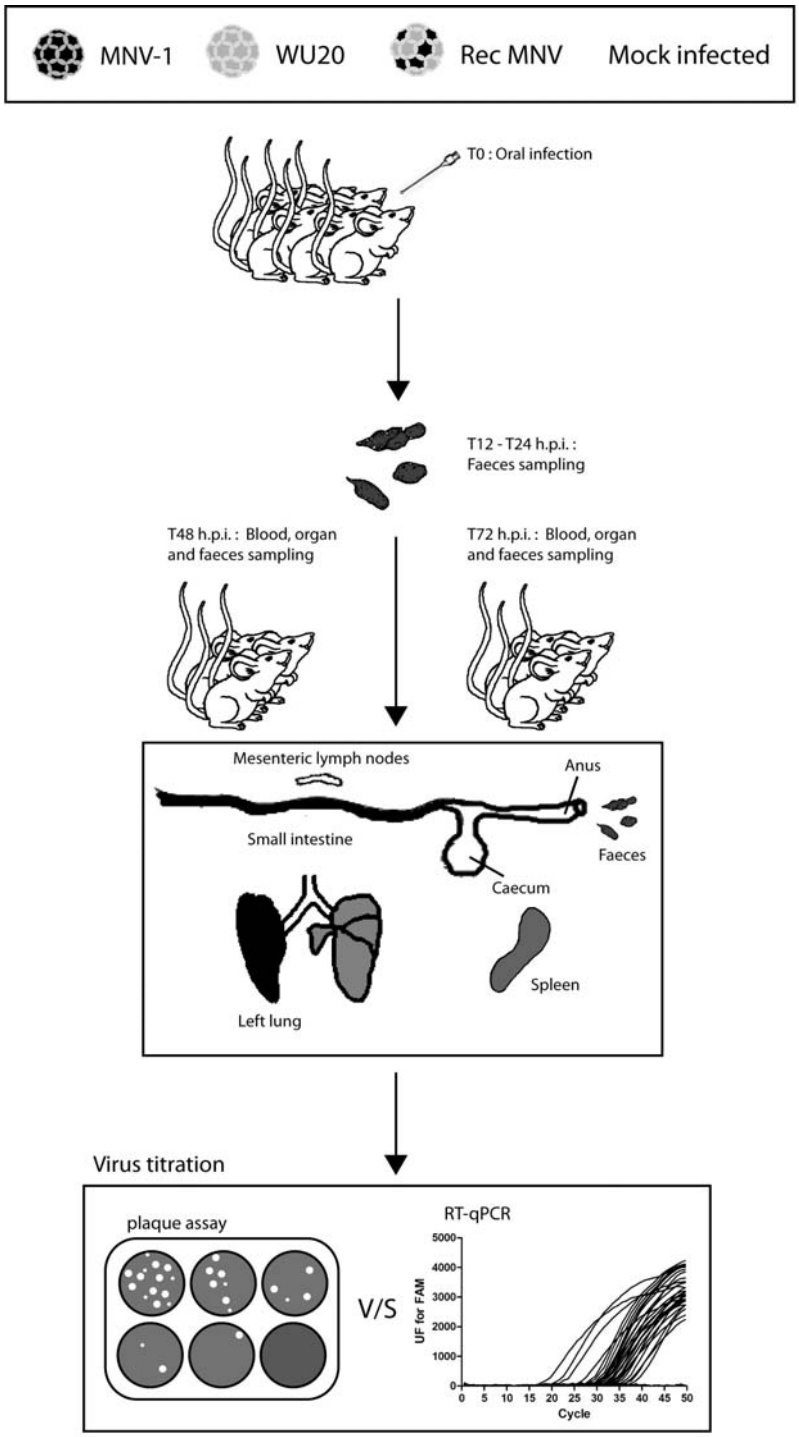

Figure 14: Schematic overview of the experimental in vivo protocol from 0 to 72 hours post infection (hpi). *Four groups of six Balb/cByJ mice were infected by oral gavage with 5.10^6 plaque forming units (pfu)/100 µL of MNV-1, WU20 or Rec MNV virus stocks. Mock-infected mice were inoculated with 100 µL of cell supernatant. Faeces were collected at 24, 48 and 72 hpi. Body weight measures, blood samples and organ tissues (small intestine, mesenteric lymph nodes, spleen and left lung) were taken for three mice in each group at time 48 and 72 hpi. Virus titres were determined in parallel by plaque assay and quantitative real time RT-PCR (RT-qPCR).*

Virus detection by RT-qPCR

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

cDNA synthesis. First-stranded cDNA was generated by an iScript cDNA Synthesis kit (Bio-Rad) according to recommendations by manufacturer.

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) was 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 number was calculated by interpolation from a standard curve. The limit of detection (LOD) was estimated at 60 cDNA copies per 100 µl of supernatant.

Preparation DNA constructs as standards.

A 469-bp PCR products for MNV-1 and WU20, including positions 6,828-7,260 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 by the QIAquick Gel Extraction Kit (Qiagen). Numbers of DNA copies were calculated based on the concentration measured by spectrophotometry (Nanodrop, Isogen) and serially diluted to defined concentrations for the elaboration of standard curves for MNV-1 and WU20 for quantification.

Virus titration and isolation by plaque assay. Virus titres were determined by plaque assay as described by Hyde et al. (2009). In order to avoid cell cytotoxicity, sample supernatants were additionally diluted 5 times for tissue samples and 15 times for blood samples. The LOD was 1 to 3 pfu/100 µl supernatant. Viruses were isolated from plaques as previously described (Mathijs *et al.*, 2010). Isolated viruses were further characterised by sequencing 300 to 600 bp stretches in 5 regions of the MNV 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).

Statistical analysis. The body weight of mice was standardised by index. Index 100 was attributed to the body weight measured at 0 dpi. The average values of each parameter were compared between Rec MNV and parental (MNV-1 and WU20) viruses by means of Welch test (Dagnelie, 1998). Because of two simultaneous comparisons were made (Rec MNV versus MNV-1 and Rec MNV versus WU20), a Bonferroni correction was applied. Statistical significance was defined as $P < 0.05/k$, with k being the number of comparisons made (e.g. $P < 0.025$). GraphPad Prism was used for graphical representations. In all graphs vertical bars indicate standard deviations of the mean values. For viral burden in organ tissues, horizontal bars represent the mean values. Asterisks represent P values inferior to $P < 0.025$.

RESULTS

Immunocompetent Balb/cByJ were orally inoculated with 5.10^6 pfu of either one of the parental viruses (MNV-1 or WU20) or Rec MNV (Figure 14). The infectious doses were confirmed to be similar by back titration using plaque assay (data not shown). Blood samples and faeces at 0 dpi were pooled and were found negative both by RT-qPCR and plaque assay. None of the infected mice showed evident clinical symptoms. At 2 dpi, the standardised average body weight of mice from Rec MNV group was significantly higher than MNV-1 and WU20 groups (P = 0.01). At 3 dpi, the standardised average body weight of Rec MNV infected mice was significantly higher than for WU20 infected mice (P = 0.003) (Figure 15).

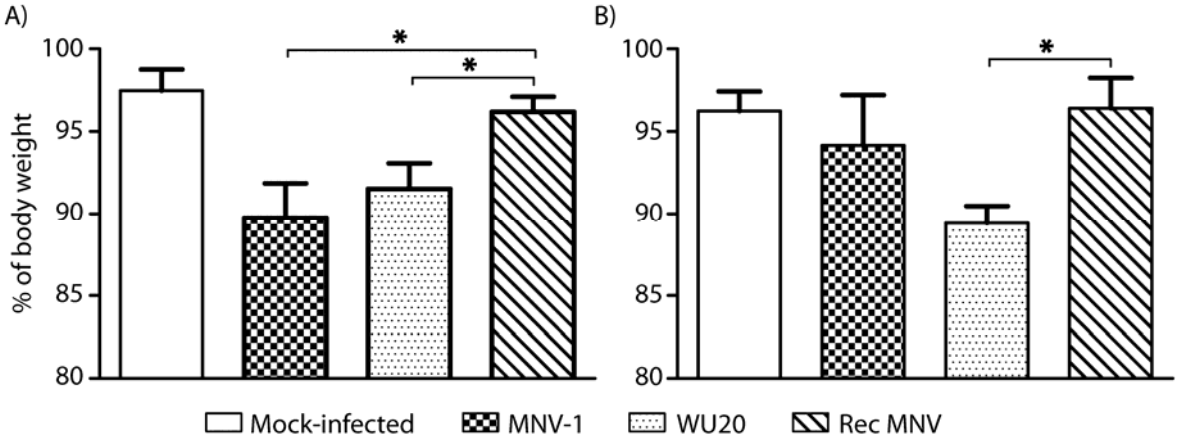

Figure 15: The recombinant MNV (Rec MNV) causes significantly lower body weight loss than the parental MNV-1 and WU20 viruses in immunocompetent mice at 48 hours post-infection. *Balb/cByJ mice were per orally inoculated with 5.10^6 pfu of either MNV-1, WU20 or Rec MNV. Mice were weighed at 0, 48 and 72 hours post-infection (hpi). Data are expressed as percentage relative to the body weight at 0 hpi and represent mean values of either A) 6 mice per group at 48 hpi or B) 3 mice per group at 72 hpi. Vertical bars show standard deviations. Statistically significant differences at P < 0.025 (*) are indicated.*

All blood samples were found negative both by RT-qPCR and plaque assay at 48 and 72 hpi. Detectable virus titres were found in faeces at 1, 2 and 3 dpi (Figure 16) and in analysed tissues for all infected mice at 48 and/or 72 hpi both by RT-qPCR and plaque assay (Figure 17). Viral loads estimated by RT-qPCR were significantly higher in the Rec MNV inoculated mice in comparison to the WU20 inoculated mice in faeces (P = 0.005) at 2 dpi (Figure 16). Higher viral loads in spleen (at 2 and 3 dpi) and MLN (at 3 dpi) were observed for MNV-1

and WU20 infected mice respectively although these differences could not be shown to be significant (P = 0.04 in both cases) (data not shown).

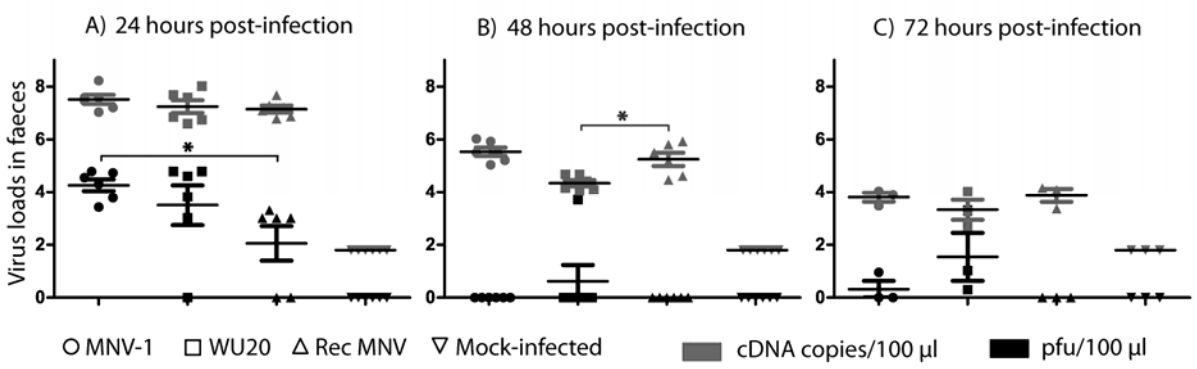

Figure 16: The recombinant MNV (Rec MNV) replicates less efficiently in comparison to parental MNV-1 virus and is rapidly cleared from faeces of immunocompetent mice. *Balb/cByJ mice were per orally inoculated with 5.10^6 pfu of either MNV-1, WU20 or Rec MNV. Faeces were collected at 0, 24 (A), 48 (B) and 72 (C) hours post-infection (hpi) and viral burdens for each virus were determined either by plaque assay (plotted in black) or quantitative real time RT-PCR (RT-qPCR) (plotted in grey). Mean values are represented by horizontal bars and vertical bars show standard deviations. Statistically significant differences at P < 0.025 (*) are shown.*

The mean viral titre in faeces obtained by plaque assay was significantly lower for Rec MNV in comparison to MNV-1 (P = 0.008) at 1 dpi (Figure 16). Furthermore, the average values for viral loads in intestine at 2 dpi (P = 0.006) and in lung at 3 dpi (P = 0.003) were significantly lower for Rec MNV infected mice than for WU20 infected mice (Figure 17). Again, at 2 dpi higher viral titres were found in intestine and lung of MNV-1 and WU20 infected mice respectively but these differences were not statistically significant (P = 0.03 and P = 0.04, respectively) (data not shown).

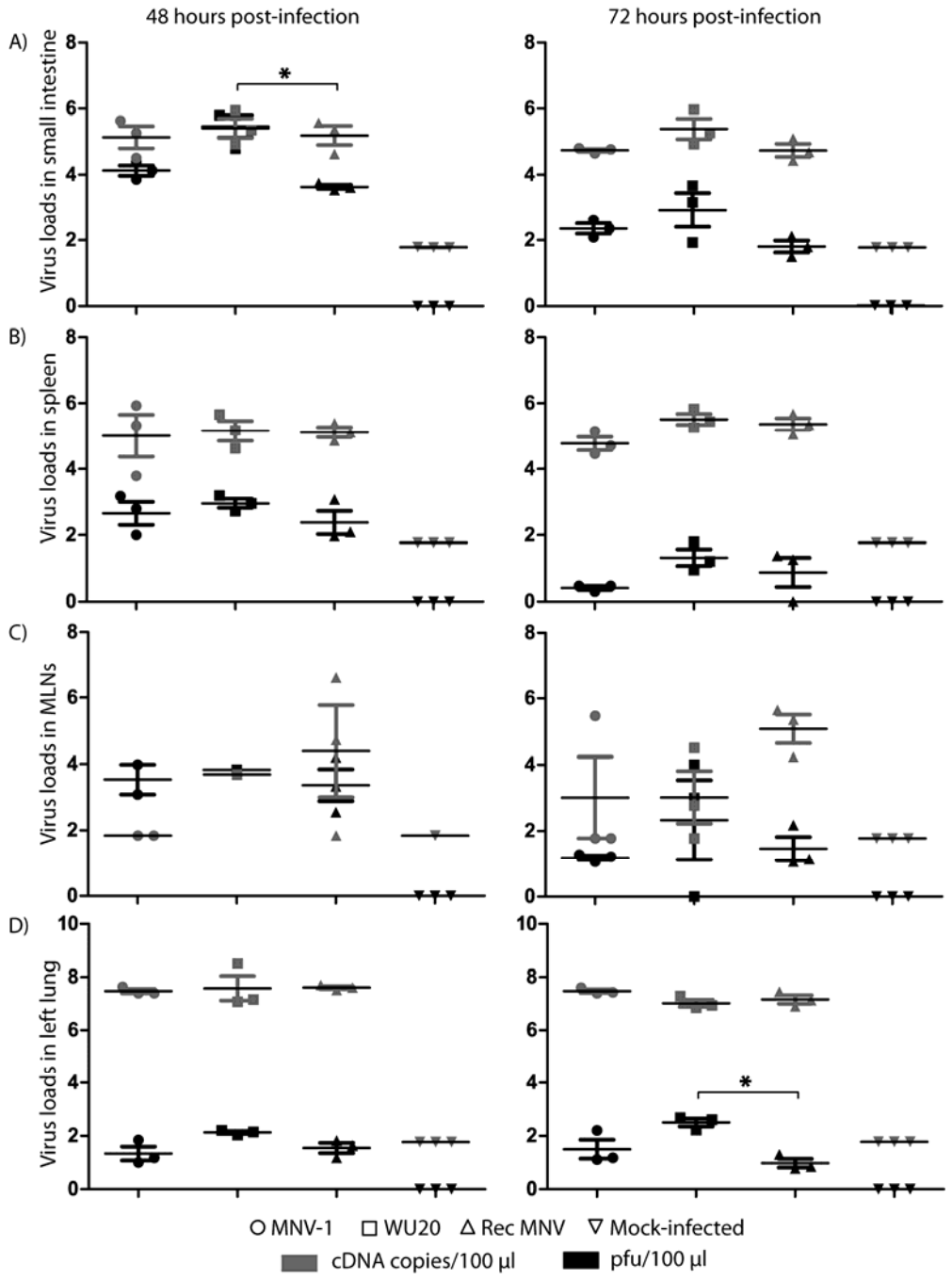

Figure 17: The recombinant MNV (Rec MNV) replicates and disseminates in a similar manner than parental MNV-1 and less efficiently than parental WU20 in immunocompetent mice. *Balb/cByJ mice were per orally inoculated with 5.10^6 pfu of either MNV-1, WU20 or Rec MNV. Organs were harvested at 48 and 72 hours post-infection. Viral loads in the small intestine (A), spleen (B), mesenteric lymph nodes (MLNs) (C) and left lung (D) were determined either by plaque assay (plotted in black) or quantitative real time RT-PCR (RT-qPCR) (plotted in grey). With the exception of the mesenteric lymph nodes, three animals were analysed per group. Mean values are represented by horizontal bars and vertical bars show standard deviations. Statistically significant differences at $P < 0.025$ (*) are shown.*

All samples were tested in parallel by RT-qPCR and plaque assay. Although most samples gave positive results with both techniques, RT-qPCR results yielded titres from 5 to 10^6 orders of magnitude higher in comparison with plaque assay results (Table 1).

Table 1. Comparison of MNV-1, WU20 and Rec MNV quantification by RT-qPCR (cDNA copies/100 µl) and plaque assay (pfu/100 µl) in various tissue samples.

Organ tissue	Minimum and maximum RT-qPCR/plaque assay
Faeces (1dpi)	6.03E+00 – 2.99E+04
Intestine	5.9E+00 – 1.32E+04
MLN	1.57E+00 – 1.11E+04
Spleen	1.46E+03 – 2.36E+04
Lung	1.42E+04 – 3.36E+06

Dpi: day post-infection; MLN: mesenteric lymph nodes

Plaque sizes of all three viruses observed from spleen supernatants were identical to those observed for viral stocks used for infection (data not shown). Moreover, sequences obtained from viruses isolated from spleens from 5 PCR fragments spanning 26.7% of the entire MNV genome did not show any substitutions between those obtained before infection (data not shown).

DISCUSSION

In this study, the virulence of an *in vitro* generated recombinant MNV (Rec MNV) was evaluated *in vivo* by comparing viral loads in faeces, blood and various organ tissues with the parental viruses (MNV-1 and WU20) in a mouse model. Body weight losses and viral loads in some organs were significantly higher for mice inoculated with the parental viruses, and more particularly WU20. These results were in line with the previous *in vitro* analysis for the Rec MNV phenotype (Mathijs *et al.*, 2010) and suggest that Rec MNV is less fit than both its parental viruses hinting that recombination could play a major role in the evolutionary mechanism of NoVs. The evaluation of body weight during the course of infection could constitute an alternative of virulence assessment in the absence of clinical signs in wild-type mice. The RT-qPCR for quantification should be used with care and should be limited to the study of viruses for which conventional titration methods are not available. Finally, this study provides the first experimental data on viral MNV strain WU20 in experimental infections, showing more efficient replication properties than the widely studied MNV-1 isolate.

Although none of the inoculated mice exhibited clinical signs, weight losses evaluated at 48 hpi for Rec MNV infected mice were significantly lower than for those infected with the parental viruses (Figure 15) and were similar to the losses observed for mock-infected mice. The fact that even mock-infected mice did lose weight could probably be attributed to the general chemical anaesthesia needed for the per oral virus inoculations. The absence of obvious clinical sign is in line with what was described for MNV-1 infections in wild-type mice for whom the STAT-1–dependent interferon innate immune response was proposed to prevent clinical disease (Goto *et al.*, 2009). Contrarily, recent studies suggest that, for similar infectious doses, MNV-1 induces subtle signs of gastroenteritis in immunocompetent hosts characterised by significant changes in internal stool volume and increased faecal inconsistency by 72 hpi (Liu *et al.*, 2009; Mumphrey *et al.*, 2007). A reduction in weight gain at 4 dpi was tentatively proposed in 129SvEv mice with mild diarrhoea but this effect failed to be significant (Mumphrey *et al.*, 2007). In the present study, in the absence of an objective measure method of faecal consistency, only macroscopic examination of faeces was performed and no variation in stool volume or consistency was observed. Nevertheless, our results suggest that MNV-1 and WU20 could be more virulent than Rec MNV by inducing a very mild gastroenteritis and/or anorexia resulting in significantly higher body weight losses in mice infected with the parental viruses. Moreover, the evaluation of body weight could be

an additional evidence for MNV disease in wild-type mice in the absence of apparent clinical signs.

In our study, no detectable virus levels were found in blood samples collected at 48 and 72 hpi. Similarly, the determination of viral burdens in sera following MNV-1 infection in wild-type 129 mice failed to detect viruses in sera throughout a 72-h course of infection (Mumphrey *et al.*, 2007). No detectable viruses were found in the lungs and viral loads present in spleen and liver were suggested to issue from a local dissemination instead of being blood borne. Again, the implication of STAT-1 in suppressing viraemia and thereby limiting the dissemination was suggested. A similar finding was reported in a chimpanzee animal model for GI and GII NoVs with virus evidence in liver tissue in the absence of detectable viraemia (Bok *et al.*, 2011). In our study, high viral loads were detected in all organs, and particularly in lungs, of mice infected with MNV-1, WU20 and Rec MNV both by plaque assay and RT-qPCR suggesting that all three infections resulted in a systemic disease. These findings do not support the hypothesis exposed by previous studies of MNV being an enteric pathogen that propagates beyond the intestine by passive dissemination. The presence of high viral loads in lungs suggests that i) viraemia occurred but was very short in time, ii) viraemia occurred at a very early stage of infection and at a very low level and of infected circulating cells or iii) viruses were transmitted through aerosols and lungs corresponded to primary sites of infection. Further experimental infection models with a higher amount of sampling periods and/or organs should be performed in order to clarify this feature.

A couple of significant differences in virus loads found by plaque assay in organs between mice infected with Rec MNV and the parental viruses suggested Rec MNV to be less replicative than the parental MNV-1 and WU20 viruses. Still, the interpretation of these results should be done carefully and variations in the experimental design such as i) the size of the mouse groups, ii) the type of mouse strains, iii) the dose of inoculation and iv) the number of cell passages before infection could interfere greatly upon the results. Mumphrey and collaborators found statistically higher detectable viral loads in proximal small intestine, spleen and liver in STAT1-/- mice infected with MNV-1 CW3 compared to CW1 whereas the differences seen in wild-type 129 mice were not statistical suggesting STAT-/- mice could be more sensitive for *in vivo* virus fitness evaluations (Mumphrey *et al.*, 2007). Using inoculation doses 1000-times lower than in our study, Karst and collaborators found detectable viral RNA levels in inbred 129 mice day 1 after MNV-1 infection in various organs (proximal small intestine, spleen and liver) but did not to detect any RNA at later times (Karst

et al., 2003). Thus, greater significant differences might have been found in organs in our study at 1 dpi. Finally, as discussed previously, the different passages in cell culture needed for the generation and the purification of Rec MNV could have been responsible for *in vitro* adaptation mutations that lead to attenuation *in vivo* (Mathijs *et al.*, 2010). In order to limit cell passages and to study NoV recombination in its natural host, it will be interesting to generate recombinants by *in vivo* co-inoculations or even create recombinant genomes by reverse genetics to be further evaluated in mice.

Previous studies based MNV titration upon either plaque assay or RT-qPCR but rarely both. In our study both methods were used in parallel and no correlation could be found between the results obtained for identical samples. Inconsistent relationships between the copy number of viral genomes and pfu have been described before (Gentilomi *et al.*, 2008) and could partly explain the great discrepancy between the experimental results of MNV infections found in the literature. Advantages of plaque assay are a low LOD and the possibility to assess the infectivity of the detected virus particles. RT-qPCR provides rapid results but requires a lot of controls to give confidence of a positive and quantitative result. Indeed extraction efficiencies vary upon the analysed matrix and the presence of PCR inhibitors may result into false negative results (A. Stals, personal communication). Therefore, the use of RT-qPCR for virulence assessments should be limited to experimental protocols where no standard plaque assay/$TCID_{50}$ methods can be implemented. Furthermore, these limitations highlight the fact that quantitative results obtained by RT-qPCR should always be interpreted with care.

The genetic stability observed through sequencing of part of the MNV genome together with the conserved plaque phenotypes for all three viruses before and after infection suggest that nucleotide substitutions were unlikely responsible for the decrease in viral fitness and virulence of Rec MNV in comparison to the parental viruses. Thus, our results suggest that the exchange of genetic material between two parental MNV viruses created a chimeric genome that exhibited a novel phenotype both *in vitro* (Mathijs *et al.*, 2010) and *in vivo*. Still, the obtaining of full genome sequences for all three viruses would be useful to confirm this finding and should exclude the occurrence of single substitutions that were previously shown to be implicated in the alteration of virus replication and virulence (Bailey *et al.*, 2010; Bailey *et al.*, 2008; Wobus *et al.*, 2004).

Results in this study suggest that the a recombination event was able to generate a viable chimeric MNV (Rec MNV) capable of infecting mice despite the fact that it was found to be less virulent in comparison to the parental genomes. Indeed, MNV recombination resulted in

the generation of a novel genetic combination that affected the biological properties of the new virus. The use of reverse genetics could be of particular interest for the creation a panel of genetic rearrangements that could be evaluated *in vitro* and *in vivo* and allowing a more accurate comprehension of the implications of recombination on the biological properties of NoVs.

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