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Favipiravir (T-705) inhibits *in vitro* norovirus replication

J. Rocha-Pereira [a,b], D. Jochmans [c], K. Dallmeier [c], P. Leyssen [c], M.S.J. Nascimento [a,b], J. Neyts [c,*]

[a] Laboratório de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira n.° 228, 4050-313 Porto, Portugal
[b] Centro de Química Medicinal da Universidade do Porto (CEQUIMED-UP), Rua de Jorge Viterbo Ferreira n.° 228, 4050-313 Porto, Portugal
[c] Rega Institute for Medical Research, University of Leuven (KU Leuven), Minderbroedersstraat 10, B-3000 Leuven, Belgium

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ABSTRACT

Human noroviruses are the primary cause of foodborne gastroenteritis. Potent and safe inhibitors are needed for the treatment/prophylaxis of norovirus infections. We demonstrate that Favipiravir [T-705, a drug in advanced clinical development for the treatment of infections with the influenza virus] inhibits *in vitro* murine norovirus replication. Time-of-drug addition studies reveal that T-705 exerts its activity at a time-point that coincides with onset of viral RNA synthesis, which is in line with the viral polymerase as the presumed target.

1. Introduction

Human noroviruses are a primary cause of gastroenteritis and are considered to be the main cause of foodborne illness throughout the world [1]. It is estimated that noroviruses are annually responsible for >200,000 deaths in developing countries [2]. The virus is gaining importance as a cause of severe childhood diarrhea as rotavirus vaccination becomes more widespread [2].

Norovirus outbreaks typically occur in nursing homes, schools, restaurants, cruise ships and hospitals. In fact, hospital outbreaks are one of the most common reasons for closure of wards [3–5]. Also, cases of chronic norovirus infections have been reported [3,6]. Given the highly infectious nature of norovirus and the extensiveness of outbreaks in semi-closed environments there is a need for prophylactic approaches. This would be particularly important for individuals with a high risk of exposure or for those that are more susceptible to complications from dehydration such as young children, elderly and immunocompromised patients. There is no vaccine or antiviral drug for the prophylaxis or treatment of norovirus-induced illness.

Noroviruses belong to the genus *Norovirus* within the family of the *Caliciviridae*. The Norwalk virus was the first norovirus to be discovered and is considered the prototype of the genus [7]. Noroviruses are a genetically diverse group of viruses, classified into five genogroups (GI-V) divided into at least 31 genetic clusters or genotypes [8]. The lack of an infectious *in vitro* model for human norovirus has largely hampered studies of norovirus biology. Thus far, cultivable caliciviruses such as the murine norovirus (MNV) or a Norwalk virus replicon-bearing cell line have been used as surrogates [9,10]. This human replicon model constitutively expresses all nonstructural proteins of the Norwalk virus. MNV is currently the only cultivable norovirus, it replicates in the murine macrophage cell line RAW 264.7 and is widely accepted as the most relevant surrogate available for human norovirus [11]. MNV is an enteric virus that causes fecal inconsistency and lethal infections in innate immunity-deficient mice [12,13]. As is the case for other (+)ssRNA viruses, noroviruses rely on the RNA-dependent RNA polymerase for amplification of the genomic RNA, which is therefore a critical enzyme for viral replication and an important antiviral target [7,14].

T-705 (favipiravir) is an antiviral molecule that is being developed for the treatment of influenza virus infections [15,16] but that exerts also activity against a number of unrelated RNA viruses, including bunyaviruses, arenaviruses [17,18] and flaviviruses [19,20].

T-705 is converted to its active form T-705 ribofuranosyl 5′-triphosphate by cellular enzymes [21]. This metabolite inhibits the influenza polymerase without affecting the synthesis of cellular RNA and DNA [22]. The exact mechanism and precise molecular interaction of the T-705 metabolite with the viral polymerase of either influenza, or the other viruses has not yet been reported. The aim of the present work was to evaluate whether the relative broad-spectrum anti-RNA virus activity of T-705 extends to noroviruses. To this end, we employed the cultivable MNV as a surrogate for human norovirus [10,11].

* Corresponding author.
E-mail address: Johan.Neyts@rega.kuleuven.be (J. Neyts).

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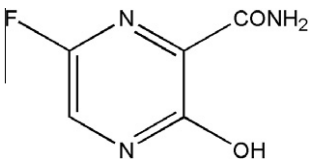

Fig. 1. Chemical structure of T-705 (favipiravir).

2. Materials and methods

2.1. Cells, viruses and compound

MNV (virus strain MNV-1.CW1) was propagated in RAW 264.7 cells grown in DMEM (Life Technologies, Gent, Belgium) supplemented with 10% or 2% FBS, 2 mM L-glutamine, 20 mM HEPES, 0.075 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 U penicillin/mL and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. T-705 (98.5% purity) was purchased from BOC Sciences (New York, USA) (Fig. 1).

2.2. Antiviral & cytotoxicity assay

The antiviral activity of T-705 was determined using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based CPE reduction assay in the MNV/RAW 264.7 cell line. To this end, RAW 264.7 cells were seeded (1×10^4 cells/well) in 96-well plates and infected with MNV at an MOI of 0,001 in the presence (or absence) of a dilution series of T-705 (3.13–200 μg/mL). Following 3 days of incubation, i.e. until complete CPE was observed in infected untreated cells, cell culture supernatants were collected for quantification of viral RNA load by quantitative RT-PCR (qRT-PCR). For the MTS reduction assay an MTS/Phenazine methosulphate (PMS) stock solution (2 mg/mL MTS (Promega, Leiden, The Netherlands) and 46 g/mL PMS (Sigma–Aldrich, Bornem, Belgium) in PBS at pH 6–6.5) was diluted 1/20 in MEM (Life Technologies, Gent, Belgium). To each well, 75 μL of MTS/PMS solution was added and the optical density (OD) was read at 498 nm 2 h later. The % CPE reduction was calculated as $[(OD_{treated})_{MNV} - OD_{VC}]/[OD_{CC} - OD_{VC}] \times 100$, where OD_{CC} represents the OD of the uninfected untreated cells, whereas OD_{VC} and $(OD_{treated})_{MNV}$ represent the OD of infected untreated cells and virus-infected cells treated with a compound concentration, respectively. The EC_{50} was defined as the compound concentration that protected 50% of cells from virus-induced CPE. Adverse effects of the molecule on the host cell were also assessed by means of the MTS-method, by exposing uninfected cells to the same concentrations of T-705 for 3 days. The % cell viability was calculated as $(OD_{treated}/OD_{CC}) \times 100$, where OD_{CC} is the OD of uninfected untreated cells and $OD_{treated}$ are uninfected cells treated with compound. The CC_{50} was defined as the compound concentration that reduces the number of viable cells by 50%. The selectivity index (SI) was calculated as CC_{50}/EC_{50}.

2.3. RNA isolation and quantitative RT-PCR

Extracellular RNA was isolated from cell culture supernatant (150 μL) using the NucleoSpin RNA Virus Kit (Macherey–Nagel, Germany), intracellular RNA was extracted from cells using the RNeasy minikit (Qiagen, Netherlands) according to the manufacturer's protocol. Forward (5′-CAC GCC ACC GAT CTG TTC TG-3′ position 4972–4991) and reverse (5′-GCG CTG CGC CAT CAC TC-3′ position 5080–5064) primers were designed for the ORF1/2 junction, as described elsewhere [23]. A 6-FAM–MGB probe was used (5′-CGC TTT GGA ACA ATG-3′ position 5001–5015). One-step qRT-PCR was performed in a 25 μL reaction mixture containing 6.25 μL One-Step Reverse Transcriptase qPCR Master Mix Plus Low ROX (Eurogentec, Belgium), 900 nM of each primer, 200 nM of probe, 0.0625 μL of RT-PCR enzyme mix and 3 μL of template MNV RNA. Cycling conditions were: reverse transcription at 48 °C for 30 min, initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min (ABI 7500 Fast Real-Time PCR System, Applied Biosystems, USA). For absolute quantification, standard curves were generated using 10-fold dilutions of MNV template DNA of known concentration.

2.4. Time-of-drug-addition assay

RAW 264.7 cells (2×10^5 cells/mL) were infected with MNV at an MOI of 0.1. After 1 h at 4 °C, cells were washed with cold (4 °C) culture media and incubated at 37 °C. To study the replication kinetics of MNV in RAW cells, cells and supernatants were harvested every 2 h until 24 h post infection (pi) and viral RNA was quantified by qRT-PCR.

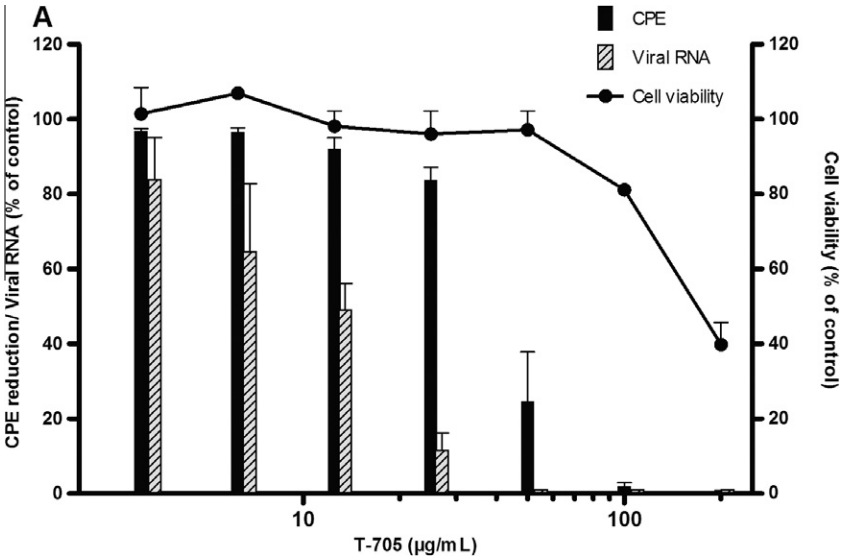

Fig. 2. Anti-norovirus activity of T-705 as quantified by: (i) virus-induced cytopathic effect (CPE) reduction assay using a colorimetric method (MTS) and (ii) measuring viral RNA levels by means of qRT-PCR. Potential cytotoxic effects were assessed in parallel. Results are mean values (± SEM) of 3 independent experiments for the CPE and RNA assay and of 2 independent experiments (± SEM) for the cytotoxicity.

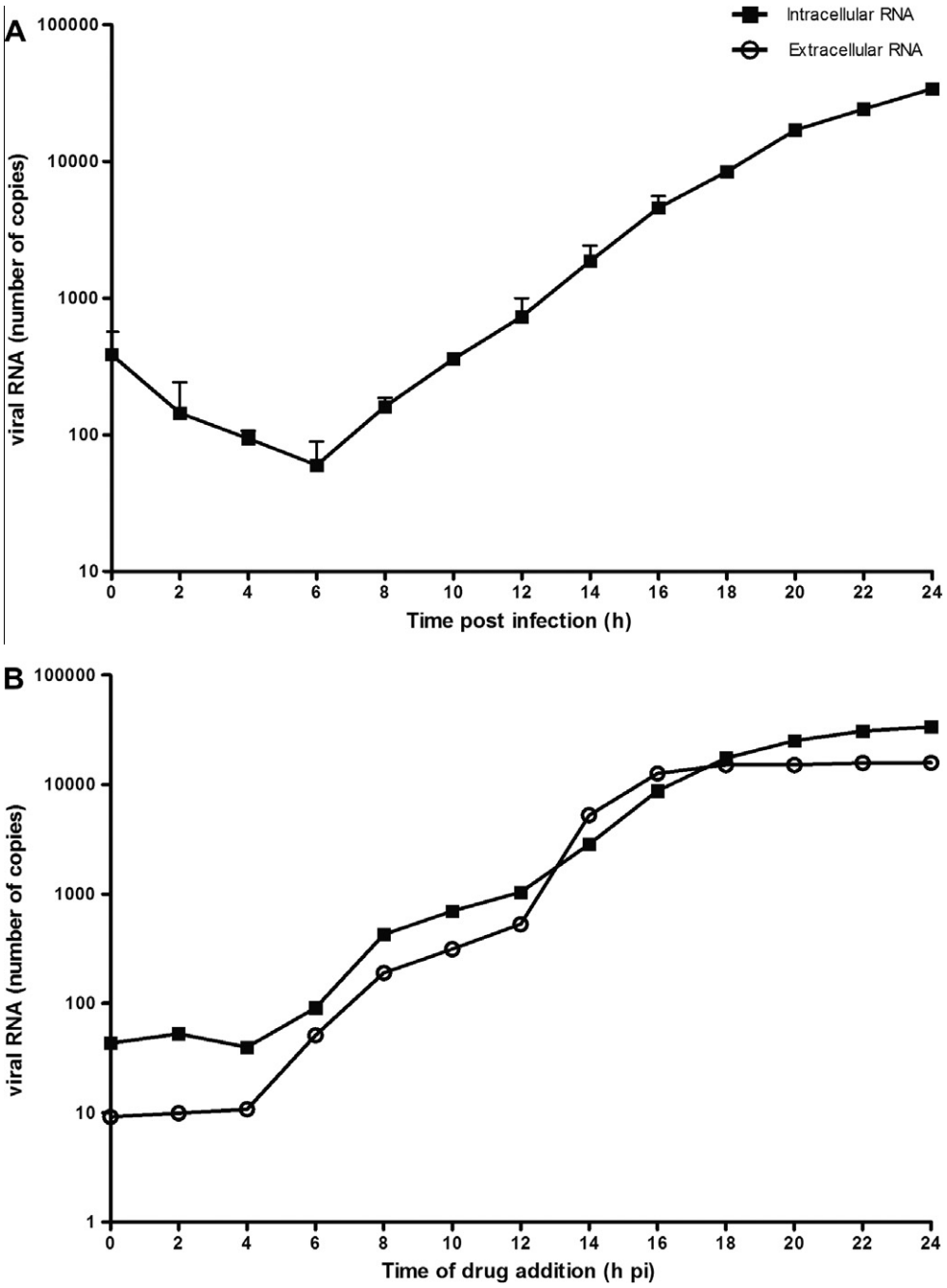

Fig. 3. Time-of-drug-addition studies of T-705 (A) Yields of intracellular (squares) viral RNA during a single replication cycle of MNV in RAW cells. Viral RNA levels were monitored at various times post infection by means of qRT-PCR. (B) Effect of time-of-(drug) addition on the antiviral activity of T-705. Extracellular and intracellular viral RNA was monitored by means of qRT-PCR at 24 h post infection (in cells treated with 100 μg/mL T-705, starting at different times post infection) and were compared with untreated infected cells.

In parallel, another set of infected cultures were treated with 100 μg/mL of T-705. The molecule was added to the infected cultures at different time points after infection (with 2 h intervals) and cultures were further incubated until 24 h pi, at which time supernatant and cells were collected separately for determination of the viral RNA by qRT-PCR.

3. Results and discussion

T-705 inhibits in a dose-dependent manner MNV-induced CPE (EC_{50}: 39 ± 4 μg/mL [250 ± 11 μM]) and MNV RNA synthesis in cell culture (EC_{50}: 19 ± 6 μg/mL [124 ± 42 μM]). Also from these experiments a CC_{50} of 171 ± 11 μg/mL was determined, indicating a selectivity index of 4.3. Despite this rather modest antiviral activity, T-705 was able to completely inhibit norovirus replication at a concentration of 100 μg/mL, which is a concentration that has little or no adverse effect on the host cell (cell viability >80%) (Fig. 2). Others have also reported that at high concentrations (up to 637 μM) T-705 did not affect the synthesis of cellular DNA or RNA [22]. Of note, since T-705 acts as a pro-drug, its cytotoxicity is expected to be cell-line dependent. Despite the fact that a comparable moderate *in vitro* antiviral activity of T-705 was also reported for flaviviruses [the yellow fever and the West Nile virus (EC_{50}: 330 and 318 μM, respectively)] this *in vitro* antiviral effect

translated in therapeutic efficacy in animal models [19,20]. It will thus be relevant to perform *in vivo* studies with MNV-infected immunodeficient mice, which could be a further step towards treatment for human norovirus infection.

We next wanted to study at which stage in the MNV replication cycle T-705 exerts its activity in. To this end, the replication kinetics of MNV in RAW cells was assessed by qRT-PCR. As can be derived from Fig. 3A, input levels of viral RNA decreased until 6 h pi, after which viral RNA levels started to increase, indicating the onset of intracellular viral RNA synthesis. Levels of extracellular viral RNA in the untreated infected cultures started to increase above background at 18 h pi (data not shown). This indicates that one replication cycle takes approximately 18 h. Addition of T-705 to the infected cultures resulted in complete inhibition of viral replication (as quantified at 24 h pi) when added during the first 4 h after infection (Fig. 3B). When added at a later time-point, i.e. just before or during onset of viral RNA synthesis, there was a gradual loss in the antiviral effect. This finding indicates that T-705 acts at a time-point that coincides with onset of viral RNA synthesis. This is in line with the proposed target for influenza, i.e. the viral RNA-dependent RNA polymerase.

In conclusion, we here show that the relative broad-spectrum anti-RNA virus activity of T-705 extends to (murine) norovirus. Once T-705 is approved for the treatment of influenza infections and in such case that we can demonstrate anti-norovirus activity of T-705 in relevant mouse models, this molecule could potentially be used for the off-label prophylaxis/treatment of human norovirus infections.

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References

[1] R.I. Glass, U.D. Parashar, M.K. Estes, Norovirus gastroenteritis, N. Engl. J. Med. 361 (2009) 1776–1785.
[2] H.L. Koo, N. Ajami, R.L. Atmar, H.L. DuPont, Noroviruses: the leading cause of gastroenteritis worldwide, Discov Med 10 (2010) 61–70.
[3] M.F. Beersma, M. Schutten, H. Vennema, N.G. Hartwig, T.H. Mes, A.D. Osterhaus, G.J. van Doornum, M. Koopmans, Norovirus in a dutch tertiary care hospital (2002–2007): frequent nosocomial transmission and dominance of GIIb strains in young children, J. Hosp. Infect. 71 (2009) 199–205.
[4] S. Hansen, S. Stamm-Balderjahn, I. Zuschneid, M. Behnke, H. Ruden, R.P. Vonberg, P. Gastmeier, Closure of medical departments during nosocomial outbreaks: data from a systematic analysis of the literature, J. Hosp. Infect. 65 (2007) 348–353.
[5] M. Koopmans, Progress in understanding norovirus epidemiology, Curr. Opin. Infect. Dis. 21 (2008) 544–552.
[6] F.H. Sukhrie, J.J. Siebenga, M.F. Beersma, M. Koopmans, Chronic shedders as reservoir for nosocomial transmission of norovirus, J. Clin. Microbiol. 48 (2010) 4303–4305.
[7] K.Y. Green, Caliciviridae: the noroviruses, in: D.M. Knipe, P.M. Howley (Eds.), Fields virology, Lippincott Williams & Wilkins, Philadelphia, 2007, pp. 949–979.
[8] D.P. Zheng, T. Ando, R.L. Fankhauser, R.S. Beard, R.I. Glass, S.S. Monroe, Norovirus classification and proposed strain nomenclature, Virology 346 (2006) 312–323.
[9] K.O. Chang, S.V. Sosnovtsev, G. Belliot, A.D. King, K.Y. Green, Stable expression of a norwalk virus RNA replicon in a human hepatoma cell line, Virology 353 (2006) 463–473.
[10] C.E. Wobus, S.M. Karst, L.B. Thackray, K.O. Chang, S.V. Sosnovtsev, G. Belliot, A. Krug, J.M. Mackenzie, K.Y. Green, H.W. Virgin, Replication of norovirus in cell culture reveals a tropism for dendritic cells and macrophages, PLoS Biol. 2 (2004) e432.
[11] C.E. Wobus, L.B. Thackray, H.W. Virgin, Murine norovirus: a model system to study norovirus biology and pathogenesis, J. Virol. 80 (2006) 5104–5112.
[12] S.M. Karst, C.E. Wobus, M. Lay, J. Davidson, H.W.t. Virgin, STAT1-dependent innate immunity to a norwalk-like virus, Science 299 (2003) 1575–1578.
[13] S.M. Mumphrey, H. Changotra, T.N. Moore, E.R. Heimann-Nichols, C.E. Wobus, M.J. Reilly, M. Moghadamfalahi, D. Shukla, S.M. Karst, Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses, J. Virol. 81 (2007) 3251–3263.
[14] M.E. Hardy, Norovirus protein structure and function, FEMS Microbiol. Lett. 253 (2005) 1–8.
[15] Y. Furuta, K. Takahashi, Y. Fukuda, M. Kuno, T. Kamiyama, K. Kozaki, N. Nomura, H. Egawa, S. Minami, Y. Watanabe, H. Narita, K. Shiraki, *In vitro* and *in vivo* activities of anti-influenza virus compound T-705, Antimicrob. Agents Chemother. 46 (2002) 977–981.
[16] R.W. Sidwell, D.L. Barnard, C.W. Day, D.F. Smee, K.W. Bailey, M.H. Wong, J.D. Morrey, Y. Furuta, Efficacy of orally administered T-705 on lethal avian influenza A (H5N1) virus infections in mice, Antimicrob. Agents Chemother. 51 (2007) 845–851.
[17] B.B. Gowen, D.F. Smee, M.H. Wong, J.O. Hall, K.H. Jung, K.W. Bailey, J.R. Stevens, Y. Furuta, J.D. Morrey, Treatment of late stage disease in a model of arenaviral hemorrhagic fever: T-705 efficacy and reduced toxicity suggests an alternative to ribavirin, PLoS One 3 (2008) e3725.
[18] B.B. Gowen, M.H. Wong, K.H. Jung, A.B. Sanders, M. Mendenhall, K.W. Bailey, Y. Furuta, R.W. Sidwell, *In vitro* and *in vivo* activities of T-705 against arenavirus and bunyavirus infections, Antimicrob. Agents Chemother. 51 (2007) 3168–3176.
[19] J.G. Julander, K. Shafer, D.F. Smee, J.D. Morrey, Y. Furuta, Activity of T-705 in a hamster model of yellow fever virus infection in comparison with that of a chemically related compound, T-1106, Antimicrob. Agents Chemother. 53 (2009) 202–209.
[20] J.D. Morrey, B.S. Taro, V. Siddharthan, H. Wang, D.F. Smee, A.J. Christensen, Y. Furuta, Efficacy of orally administered T-705 pyrazine analog on lethal west nile virus infection in rodents, Antiviral Res. 80 (2008) 377–379.
[21] Y. Furuta, K. Takahashi, K. Shiraki, K. Sakamoto, D.F. Smee, D.L. Barnard, B.B. Gowen, J.G. Julander, J.D. Morrey, T-705 (favipiravir) and related compounds: novel broad-spectrum inhibitors of RNA viral infections, Antiviral Res. 82 (2009) 95–102.
[22] Y. Furuta, K. Takahashi, M. Kuno-Maekawa, H. Sangawa, S. Uehara, K. Kozaki, N. Nomura, H. Egawa, K. Shiraki, Mechanism of action of T-705 against influenza virus, Antimicrob. Agents Chemother. 49 (2005) 981–986.
[23] L. Baert, C.E. Wobus, E. Van Coillie, L.B. Thackray, J. Debevere, M. Uyttendaele, Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure, Appl. Environ. Microbiol. 74 (2008) 543–546.