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RESEARCH

Biological risk assessment related to intracerebral injection of TMEV-DA

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Abstract

Theiler's murine encephalomyelitis virus (TMEV) is a natural enteric pathogen of mice that, when injected intracranially, induces a demyelinating disease resembling multiple sclerosis. However, data on viral shedding following intracranial infection are sparse for some strains and entirely absent for others, such as the Daniels strain, making it difficult to accurately assess the biological risks associated with infected animals. In our study, we used a combination of RT-qPCR and immunoassays to evaluate viral shedding and the associated infectious risk. We show that viral RNA is detectable in the feces of some mice and that the proportion of shedding animals varies by mouse line. Nevertheless, none of the sentinel mice exposed to soiled bedding from infected animals exhibited seroconversion, indicating that the viral particles are either present at levels below the minimum infectious dose or represent residual, non-infectious material rather than active, transmission-competent virus.

Background

Theiler's murine encephalomyelitis virus (TMEV) is a natural enteric pathogen of mice, classified within the genus *Cardiovirus* of the family *Picornaviridae*. This virus is non-enveloped and has a genome consisting of a positive-sense, single-stranded RNA molecule encoding a single polyprotein. The later begins with a short leader (L) peptide and is followed by 11 additional gene products arranged in the characteristic *L–4–3–4* pattern typical of picornaviruses [1, 2].

TMEV is transmitted via the fecal-oral route. In most cases, natural infection with Theiler's virus in adult mice results in an asymptomatic enteric infection. Only rarely does the virus cross the blood-brain barrier (BBB) and causes demyelinating lesions, with an estimated incidence of one in 4,000 to 10,000 cases [3, 4. In contrast, intracranial (IC) inoculation of the virus causes neurologic lesions in a high proportion of individuals. Accordingly, TMEV strains are classified into two distinct genetic subgroups, which differ in their neurovirulence as observed in experimental models following IC inoculation: (1) a highly virulent subgroup, including the GDVII and FA strains, which causes acute and often fatal polioencephalomyelitis in mice, (2) a less virulent subgroup, known as Theiler's original (TO) group, including the Daniels (DA) and BeAn strains, which lead to persistent infection of the central nervous system (CNS) in permissive mouse lines. This infection is characterized by mononuclear cell infiltration of the leptomeninges and CNS white matter, along with primary demyelination [5] known as TMEV-induced demyelinating disease (TMEV-IDD).

TMEV-IDD is a well-established reference model of virus-induced demyelination and is widely used to study multiple sclerosis (MS) [6, 7. Mouse strains differ markedly in their susceptibility to TMEV-IDD: highly susceptible strains such as SJL, SWR, and DBA/2 develop persistent CNS infection and progressive demyelinating disease, whereas resistant strains such as C57BL/6 and BALB/c clear the virus within a few weeks [2, 8 [Yamada et al. 1991; Gerhauser et al. 2019]. Among the TO subgroup strains, the DA strain has a higher propensity to induce demyelination in susceptible mice, while the BeAn

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strain elicits more robust antibody responses [8, 7][Pike et al. 2022; Gerhauser et al. 2019]. Beyond its use as a model of MS, TMEV infection has also been employed to model viral encephalitis-induced epilepsy: in C57BL/6 mice, intracerebral inoculation with the BeAn strain leads to hippocampal inflammation and seizures rather than demyelination, providing a distinct model of epilepsy [2]. Since the first utilization of TMEV as mouse model for neuropathies in the early 1930 s [10, 11, our awareness on biohazard risk potentially linked to the usage of viruses has progressively increased. Following IC inoculation with the BeAn strain in SJL mice, viral RNA has been shown to be undetectable in serum, saliva and feces throughout both the acute and chronic phases of infection [12] [Modica et al. 2016]. However, results obtained with a given virus strain in a specific mouse line cannot always be extrapolated to other virus or mouse strains. Although infectious RNA has been detected in the spinal cords of mice chronically infected with the TMEV-DA strain [13], there is currently no data on viral shedding following IC inoculation with this strain. As a result, there is no solid scientific basis for accurately assessing the biological risk associated with these infected animals or for defining the appropriate biosafety measures to apply.

To address this gap, we conducted a two-pronged investigation: first, by using RT-qPCR to detect the presence of viral RNA in the feces of IC infected animals; and second, by exposing sentinel mice to potentially contaminated bedding to determine whether any viral particles present in the feces were infectious.

We detected low levels of viral RNA in the feces of all SWR/J TMEV-infected mice and ~15% of SJL/JRj TMEV-infected mice; however, none of the sentinel mice showed evidence of seroconversion. These findings indicate that the virus administered via IC injection can indeed be shed in the feces, albeit most likely in a non-infectious state.

Methods

Animals and housing

Twelve SJL/JRj, 12 SWR/J, and 24 DBA/2JRj female mice were used in this study. SJL/JRj and SWR/J mice were selected because both strains are susceptible to TMEV-IDD, but display distinct disease courses: while both strains develop gait alterations and reduced exploration following IC infection, SJL/JRj mice exhibit an earlier and more severe decline in spontaneous locomotion compared to SWR/J mice [6]. Including both strains therefore allowed us to assess whether shedding patterns differ between animals with distinct neurological disease progression. DBA/2JRj mice were used as sentinels because they are a well-characterized inbred strain, available under SPF conditions, and suitable for standardized microbiological monitoring in animal facilities. Furthermore, inbred DBA mice have been noted to be more likely to develop clinical disease when infected with certain pathogens, making them potentially preferable when increased detection sensitivity is desired [14]. All procedures were approved by the University of Liège Animal Ethics Committee (protocol numbers #20–2295 and #20–2297). SJL/JRj and DBA/2JRj mice were obtained from Janvier Laboratories and delivered at 4 and 6 weeks of age, respectively. SWR/J mice were bred in-house.

Animals were maintained in an A2 biosecurity-level facility under standard housing conditions: temperature between 21 °C and 24 °C, relative humidity of 40–60%, and a 12-hour light/dark cycle. They had ad libitum access to standard rodent chow and tap water. Prior to infection, mice were housed in groups of six in individually ventilated cages (IVCs, Tecniplast). After infection, they were housed individually to enable monitoring of each animal and to prevent potential oro-fecal transmission, should intracranially injected virus be shed in feces. Individual mice were identified using ear tags.

Sentinel DBA/2JRj mice were exposed to bedding soiled by either SJL/JRj or SWR/J mice that had received intracranial (IC) injections of the TMEV-DA strain (12 mice), or by mock-infected control mice (12 mice). Each sentinel mouse was paired with a specific donor mouse and was exposed exclusively to that individual's soiled bedding throughout the duration of the experiment. In practice, infected mice were housed in cages equipped with disposable cage liners (Tecniplast). The liners, along with bedding and enrichment materials (e.g., cotton nesting material and wooden shelters), were replaced weekly. The soiled liners, including all bedding and enrichment items, were then transferred to the corresponding sentinel cages. The infected mice were monitored over a period of three months following intracranial (IC) injection. The sentinel mice where exposed to their soiled bedding during the same period. All mice were monitored daily by visual observation during the entire experimental course, i.e. until 12 weeks p.i. Since clinical signs of TMEV-IDD, such as hindlimb paresis and spastic paralysis, typically do not appear within this time window, no formal clinical scoring was performed.

Induction of TMEV-IDD and fecal sampling

At five weeks of age, 6 SJL/JRj and 6 SWR/J mice were intracerebrally injected with 2×10^6 plaque-forming units (p.f.u.) of the TMEV-DA strain suspended in DMEM. An additional 6 age-matched mice from each strain received an equal volume of DMEM alone and served as mock-infected controls. Intracranial injections were performed under deep isoflurane anesthesia in a biosafety cabinet. Anesthesia was induced by placing mice in an induction chamber with 5% isoflurane until a slow, bradypneic respiratory pattern was observed. Mice were then positioned in ventral recumbency and anesthesia was maintained via a nose cone delivering approximately 3% isoflurane. The injection site was located midway between the eye and the ear, lateral to the midline. The injection was performed using an insulin syringe (0.3 mL) fitted with a needle stop to limit penetration depth and ensure consistent intracranial placement. The needle was slowly withdrawn after injection to minimize solution leakage. Following the procedure, each mouse was housed individually in a clean home cage and monitored until full recovery from anesthesia.

Fresh fecal samples from TMEV- and mock-infected mice were collected immediately before intracerebral (IC) injection, at 1 and 3 days post-infection (p.i.), weekly from week 1 through week 4 p.i., then every second week until week 12. To ensure sample freshness, feces were collected during cage cleaning sessions. Mice were first transferred to clean cages, and at least 5 freshly excreted fecal pellets were collected from each mouse. Samples were then flash-frozen in liquid nitrogen and stored at −80 °C until further analysis.

RNA extraction and virus amplification

Total RNA was extracted on 2–3 fecal pellets with TRIzol reagent (Invitrogen, Carlsbad, CA, USA.) according to the manufacturer's protocol. Viral particle quantification was performed with the following primers and probe: TMEV.F7b (CTTTGGATTCAAGGCAACACC), TMEV.R4 (GCCAGGGAAAGAAGAGAGTC), and TMEV.Probe 7 (/56-FAM/CGCTTCTGT/ZEN/CCGGATCAGGTACAA/3IABkFQ/). PCR reactions were conducted in triplicate in a 15 µL volume containing 1× Luna Universal Probe One-Step Reaction Mix, 1× Luna WarmStart RT Enzyme Mix, 0.4 µM of each primer, 0.2 µM of probe, and 37.5 ng of RNA. Amplification was performed on a StepOnePlus (Applied Biosystems) under the following conditions: 10 min at 55 °C, 1 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 1 min at 60 °C. Viral RNA extracted from an infected cell culture and corresponding to 1000 p.f.u. serves as a positive amplification control. Ten µl of PowerUp™ SYBR™ Green (Applied Biosystems) were added to all positive wells after amplification, before the samples were returned to the qPCR machine to perform a melting curve analysis. GAPDH amplification was performed on all samples to confirm RNA quality. The RT-qPCR was conducted with the same protocol as for the viral amplification but with the following primers and probes: mouse.GAPDH.F (GTGGAGTCATACTGGAACATGTAG), mouse.GAPDH.R (AATGGTGAAGGTCGGTGTG), and mouse.GAPDH.probe (/56-FAM/TGCAAATGG/ZEN/CAGCCCTGGTG/3IABkFQ).

ELISA and MIA

Infected mice were monitored for a period of three months. At the end of this period, sentinel mice were submitted to QM Diagnostics. Anti-TMEV antibodies were assessed by in-house developed and validated ELISA and MIA assays using GDVII strain antigen, with demonstrated cross-reactivity to other TMEV strains, performed by QM Diagnostics (Nijmegen, The Netherlands) under ISO/IEC 17,025 accreditation.

Results

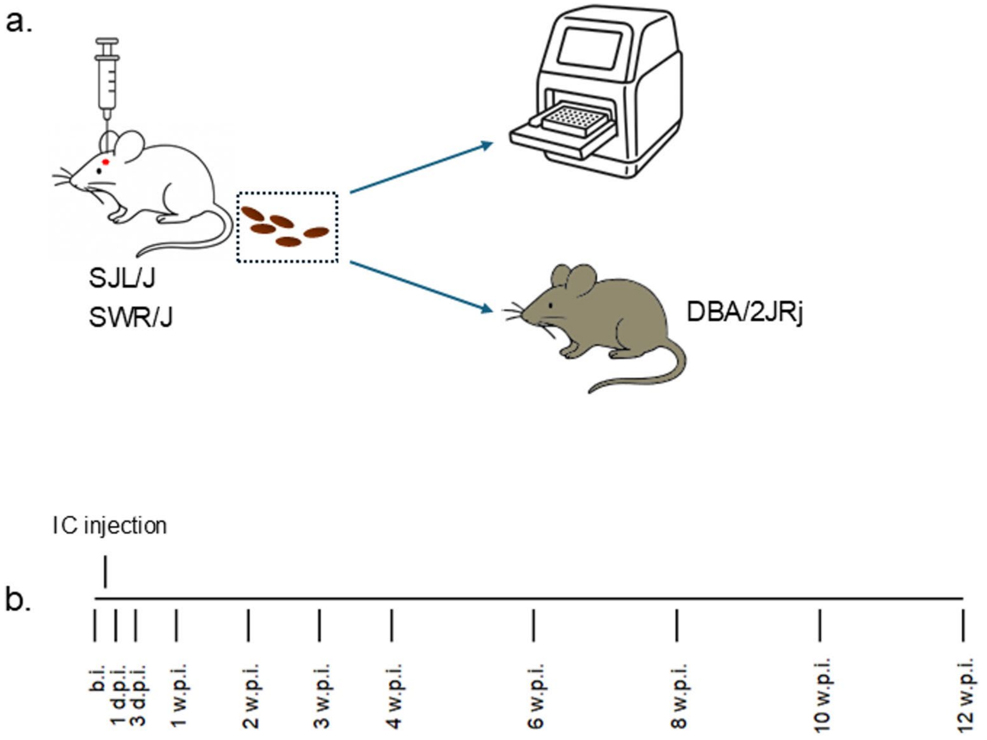
Experimental design

Twelve SJL/JRj mice and 12 SWR/J mice were randomly assigned to infectious or control group. Hence leading to the constitution of four experimental groups of 6 mice:

- TMEV infected SJL/JRj ($^{\text{TMEV}}$ SJL/JRj).
- TMEV infected SWR/J ($^{\text{TMEV}}$ SWR/J).
- Mock-infected SJL/JRj ($^{\text{Mock}}$ SJL/JRj).
- Mock-infected SWR/J ($^{\text{Mock}}$ SWR/J).

Each mouse was housed individually after the IC injection to prevent cross-contamination, for example, by licking the inoculation site of another mouse immediately after the IC injection or by oro-fecal transmission on a later timepoint, should intracranially injected virus be shed in feces. One $^{\text{TMEV}}$ SWR/J mouse died the day after infection, hence reducing its group size to five. Fecal pellets were collected from each individual immediately before IC injection and at 10 time points after infection (Fig. 1), quick frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Twenty-three sentinel DBA/2JRj mice were paired to one of the SJL/JRj or SWR/J mouse and exposed to its soiled bedding over a 3-month period (Fig. 1).

Fig. 1 Experimental design and timeline. (**a**) IC injection of SJL/JRj and SWR/J mice, followed by a two-pronged investigation: fecal sampling to collect shed RNA for RT-qPCR analysis and exposure of sentinel DBA/2JRj mice to feces of infected SJL/JRj or SWR/J mice to check for the presence of infectious virus. (**b**) Timeline indicating the sampling times relatively to the IC injection. b.i. = before infection, d.p.i. = days post infection, w.p.i. = weeks post infection



Detection of viral RNA in the feces of IC-infected SJL/JRj and SWR/J mice using RT-qPCR

Fecal samples were collected at 11 time points: before infection, 1 and 3 days post-infection (p.i.), and at 1, 2, 3, 4, 6, 8, 10, and 12 weeks p.i. Total RNA was extracted from fecal samples and analyzed by RT-qPCR to detect the presence of TMEV RNA. A second RT-qPCR targeting GAPDH, served as an internal control for RNA quality. RT-qPCR primers and probes were similar to the ones used by Modica et al. [9] in their study of BeAn TMEV shedding, with adaptations to the Daniels strain sequence. They were tested on RNA purified from crude infected BHK-21 cell extract and gave very clear and high amplification. Feces are known to be a complicated matrix to work with. They may, in particular, contain inhibitors preventing proper PCR amplification. To rule out this potential issue, we compared amplification from RNA derived from infected BHK-21 cells, either alone or combined with RNA extracted from uninfected mouse feces, across concentrations ranging from 1 to 10^6 plaque forming unit (p.f.u.) (Fig. 2). Ct values were slightly higher (mean = 0,2) upon addition of feces RNA. This very small increase should not compromise our ability to detect TMEV in feces of infected mice if it is shed.

Specificity of amplified PCR products was confirmed by melt curve analysis. No specific amplification was detected in any mouse before infection, or in any mock-infected mice at any time point (Fig. 3a). Among the infected animals, viral RNA was detected in the feces of all five $^{\text{TMEV}}$ SWR/J mice at one or more time points, and in one of the six $^{\text{TMEV}}$ SJL/JRj mice (Fig. 3a, b). Three of $^{\text{TMEV}}$ SWR/J mice shed RNA at three consecutive time points (3 d.p.i., 1 w.p.i., and 2 w.p.i.). The only $^{\text{TMEV}}$ SJL/JRj sheding mouse was positive at two non-sequential time points (1 w.p.i., and 4 w.p.i.). Noteworthy, sheding was always low with Ct values ranging from 29.3 to 38.

Assessment of the infectivity of TMEV-DA viral particles potentially present in feces

The infectivity of TMEV-DA viral particles potentially shed in the feces was assessed using DBA/2JRj sentinel mice. DBA/2JRj mice were exposed to bedding soiled by either SJL/JRj or SWR/J mice that had received an IC injection of the TMEV-DA strain ($n=11$), or by mock-infected control mice from the two same lines ($n=12$), as detailed in the Materials and Methods section. Sentinel mice were continuously exposed to freshly soiled bedding throughout the entire three-month period following IC injection (including the acute phase of infection) with bedding replaced weekly. At the end of this period, serum samples were submitted to QM Diagnostics for serological testing.To assess seroconversion, the presence of anti-TMEV antibodies was evaluated using MIA and ELISA. Employing both platforms minimizes the likelihood of false positives or negatives and provides cross-validation of results. While MIA offers higher sensitivity and a broader dynamic range, ELISA

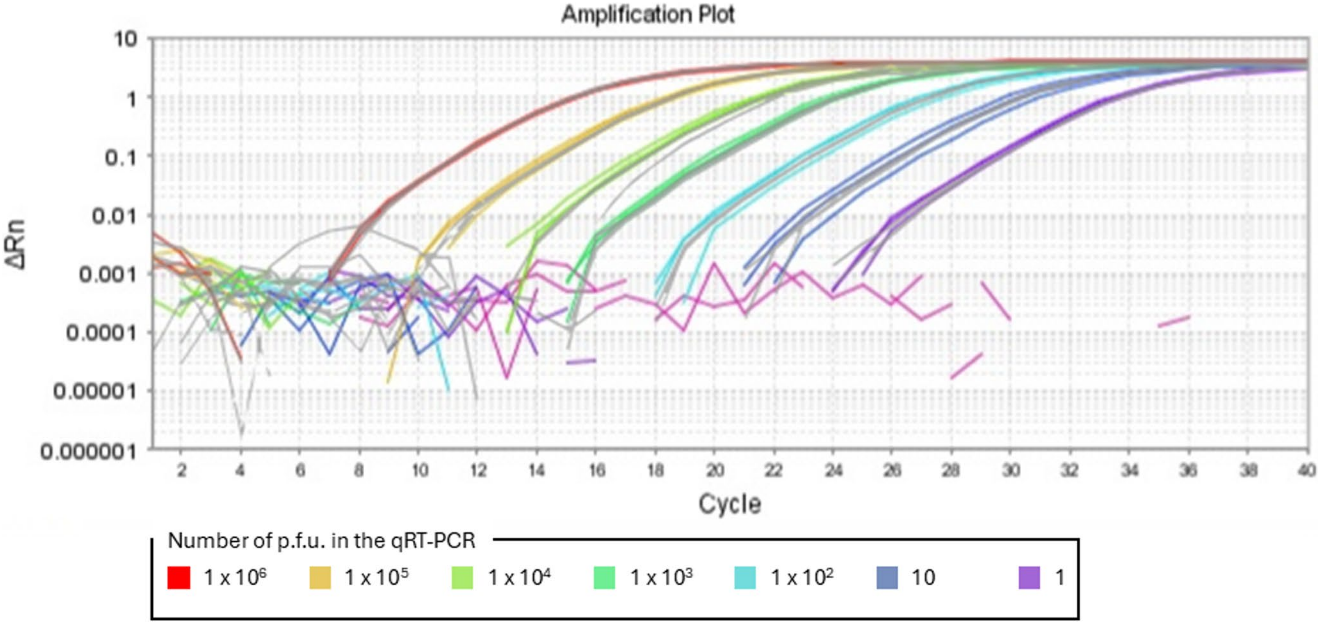


Fig. 2 Amplification plot showing the slight increase in Ct when fecal RNA is added to TMEV infected BHK-21 RNA. Colored curves = BHK-21 RNA alone, grey lines = BHK-21 RNA combined with fecal RNA. Number of p.f.u. engaged in the PCR reactions is color coded according to the legend below the amplification curves

may provide enhanced specificity. The assays used by QM Diagnostics are based on antibodies raised against the GDVII strain of TMEV. These tests are also appropriate for detecting antibodies against the DA strain, as confirmed by QM Diagnostics and supported by the fact that all TMEV isolates are antigenically closely related and belong to a single serogroup [15].

No evidence of seroconversion was detected in any of the sentinel mice by either MIA or ELISA (Table 1). We hence concluded that the viral particles detected in the feces of the 6 intracranially TMEV-infected shedding mice are non-infectious.

Discussion

RT-qPCR is a rapid, sensitive, and specific detection method and it has emerged as the gold standard for virus detection and quantification [16]. However, it cannot distinguish between infectious and non-infectious viruses and is therefore not sufficient to determine transmissibility risk. In this study, we combined RT-qPCR with the use of sentinel mice to address the dual question of the presence and infectivity of TMEV in the feces of intracranially infected mice.

We confirmed the presence of TMEV-RNA in the feces of all $^{\text{TMEV}}$ SWR/J and one out of six $^{\text{TMEV}}$ SJL/JRj mouse (~16.5%). Hence shedding of TMEV-DA viral RNA appears to be line-dependent. This host-strain effect parallels observations from other murine viral infections [17–19]. Interestingly, Modica et al. [12] reported that fecal shedding of TMEV was not detected in IC-inoculated SJL mice infected with the BeAn8386 strain, as none of the fecal samples tested positive by RT-qPCR. These contrasting findings in SJL mice suggest that TMEV RNA shedding may also be viral-strain dependent. Notably, the viral RNA identified in our study showed no evidence of infectious potential, as none of the sentinel mice exposed to the fecal material developed an immune response against TMEV. This suggests that although viral RNA can be transiently shed in feces following IC infection, the viral particles are either present at levels below the minimum infectious dose or represent residual, non-infectious material rather than active, transmission-capable virus. The mechanisms by which TMEV-DA RNA reaches the feces following IC inoculation remain to be elucidated. Several pathways can be hypothesized. First, following IC injection, the BBB is transiently disrupted at the injection site, potentially allowing viral particles to enter the bloodstream directly. From there, the virus could reach the gastrointestinal tract via systemic circulation and be shed in feces. Second, viral particles could exit the CNS via the cerebrospinal fluid, enter the lymphatic system through the arachnoid villi, and subsequently reach the peripheral circulation and the intestinal tract. In particular, infected macrophages, which are known to harbor TMEV in the chronically infected CNS, could serve as a cellular vector for viral dissemination via this route.

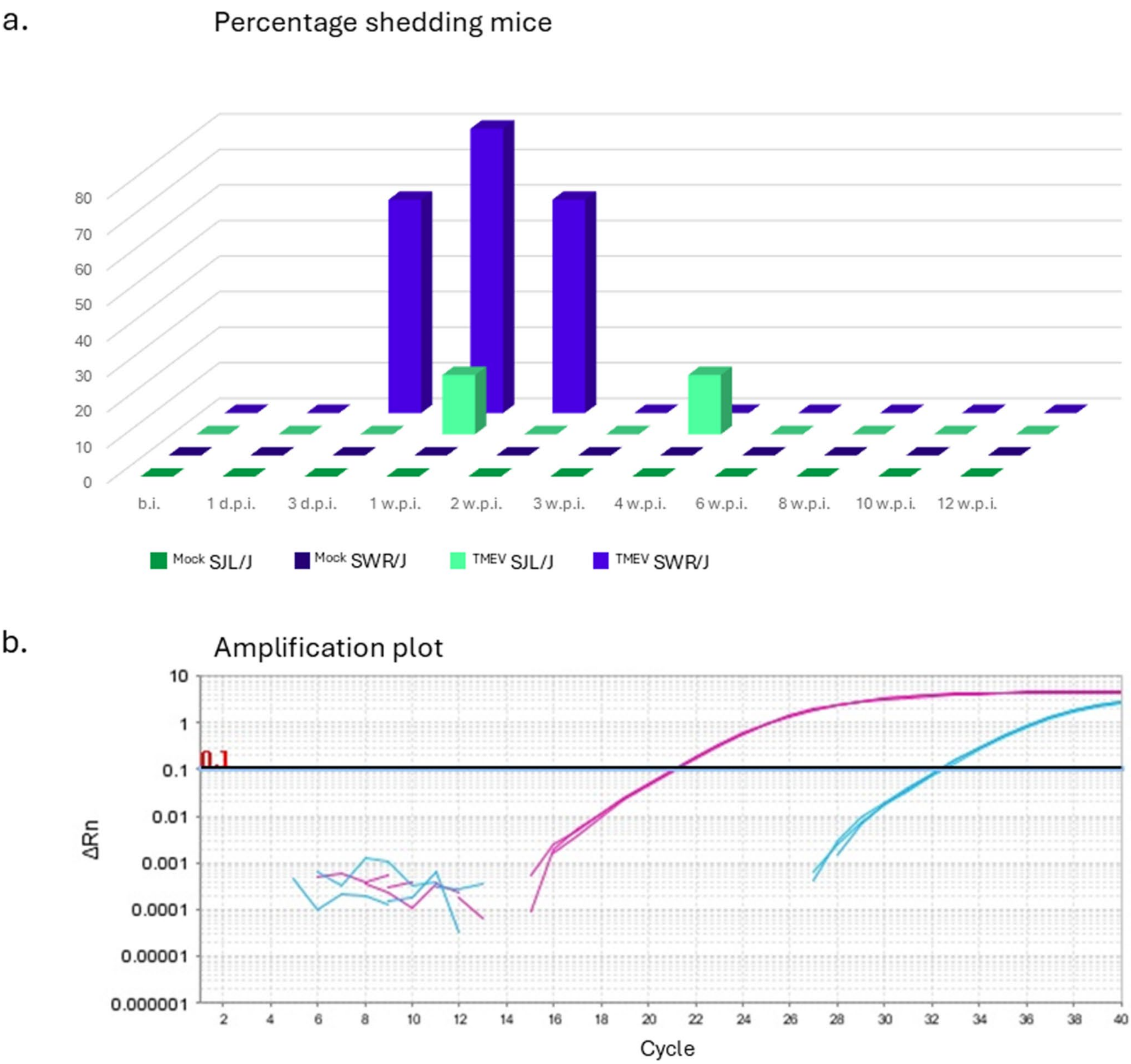


Fig. 3 Detection of TMEV-DA RNA in mouse feces by qRT-PCR. (**a**) Percentage of mice shedding TMEV-DA RNA in fecal samples over time. b.i = before infection, d.p.i. = days post-infection, w.p.i. = weeks post-infection. Note that the figure displays the proportion of shedding animals at each individual time point. Since shedding was transient and not synchronized across individuals, the cumulative proportion of shedding animals over the entire experiment (100% of TMEV SWR/J mice) exceeds the maximum value observed at any single time point. (**b**) Amplification plot from a fecal samples collected at 1 week *p.i.* A clear amplification signal crossing the threshold (black horizontal line) is observed after ~ 32 cycles in one infected SWR/J mouse (light blue), indicating viral RNA shedding. A strong amplification curve is also present in the positive control (pink). Samples were run in triplicate

Of note, the possibility of grooming-related contamination is highly unlikely, as each mouse was individually housed in a clean cage immediately after injection.

Importantly, the timing of fecal shedding provides valuable clues regarding the most likely mechanism. In SWR/J mice, viral RNA was detected exclusively during the acute phase of infection, peaking at 2 weeks post-infection and becoming undetectable more than 4 weeks post infection. This temporal pattern closely coincides with the period of maximal viral replication in neurons and transient BBB disruption following IC injection, strongly suggesting that a transient viremia during the acute phase is the primary route by which viral particles reach the gastrointestinal tract. In contrast, a mechanism involving infected macrophages trafficking out of the CNS would be expected to produce shedding during the chronic phase also, which

Table 1 MIA and ELISA results for the 23 DBA/2JRj sentinel mice enrolled in this study. No evidence of seroconversion was detected in any of the sentinel mice. Paired line indicates if the considered sentinel mouse was exposed to the soiled bedding of a SJL/JRj or SWR/J mouse. Viral status indicates if the considered sentinel mouse was exposed to the bedding of a virus- or mock-infected mouse. Nbr mice corresponds to the number of sentinel mice tested for the specific category. - = negative reaction

Paired line	Viral status	Nbr mice	ELISA	MIA
SJL/JRj	Mock	6	-	-
SJL/JRj	TMEV	6	-	-
SWR/J	Mock	6	-	-
SWR/J	TMEV	5	-	-

was not observed here. These observations therefore point towards direct hematogenous dissemination during the acute phase as the most plausible explanation.

Distinguishing definitively between these hypotheses will require additional investigations. Direct examination of intestinal tissue by RT-qPCR and immunohistochemistry at multiple time points following IC inoculation would determine whether the virus actually infects the intestinal mucosa or merely transits through the digestive tract. Analysis of blood and CSF samples by RT-qPCR at early time points post-infection would help assess whether viremia or CSF viral load correlates with fecal shedding.

The strain-dependent pattern of fecal viral RNA shedding observed in this study (with SWR/J mice shedding more consistently than SJL/JRj mice) likely reflects differences in viral replication dynamics between these two mouse strains during the acute phase of infection. It is well established that different inbred mouse strains display markedly different susceptibility profiles to TMEV infection, and that these differences are largely genetically determined, particularly through MHC class I genes which influence viral clearance efficiency [20]. While both SJL/JRj and SWR/J mice are considered susceptible to TMEV-IDD, they differ significantly in their disease course, remyelination capacity and axonal pathology [6, 21

From a biosafety perspective, the results of this study provide useful, albeit preliminary, guidance for the management of IC-infected mice in laboratory animal facilities. Our data consistently show that fecal shedding of TMEV-DA RNA is restricted to the acute phase of infection, with viral RNA detectable up to 4 weeks post-infection in individual animals. Critically, none of the sentinel DBA/2JRj mice exposed to soiled bedding throughout the entire study period developed anti-TMEV antibodies, suggesting that the shed viral material was not infectious under the conditions tested. Taken together, these findings suggest that the biological risk associated with IC-inoculated TMEV-DA mice is limited, and is primarily concentrated during the first weeks following injection.

Based on these observations, biosafety precautions may be relaxed from 6 weeks p.i., as this represents the first time point at which viral RNA was no longer detectable in the feces of any animal in our study and the risk of viral transmission through the fecal-oral route appears negligible. Concretely, infected mice should be housed in cages equipped with a cage liner, which should then be disposed of directly as class B2 biological waste, and water bottles should be autoclaved before washing. Given the limited number of animals included in this study, these recommendations should be regarded as preliminary. Studies involving larger cohorts of animals, including additional mouse strains and longer follow-up periods, will be needed to consolidate these biosafety guidelines and to determine whether the absence of seroconversion in sentinel mice holds across a broader range of experimental conditions.

Conclusions

This study demonstrates that TMEV-DA RNA can be detected in feces of intracranially infected animals at a a line-dependent frequency with SWR/J mice shedding more consistently than SJL/JRj mice. Importantly, the absence of seroconversion in sentinel DBA/2JRj mice exposed to soiled bedding throughout the entire study period indicates that the shed viral material was not infectious under the conditions tested, suggesting that the biological risk associated with IC-inoculated TMEV-DA mice is low. Current evidence indicates that the detected RNA likely represents either residual, non-infectious material or infectious particles present below the threshold required for productive infection. Based on these findings, we recommend that biosafety precautions be relaxed after 6 weeks p.i. However, given the limited number of animals studied, these conclusions should be regarded as preliminary, and further studies involving larger cohorts and additional mouse strains are warranted to consolidate these biosafety recommendations.

Abbreviations

BBB	Blood-brain barrier
IC	Intracranial
CNS	Central nervous system
$^{\text{Mock}}$ SJL/JRj	Mock-infected SJL/JRj
$^{\text{Mock}}$ SWR/J	Mock-infected SWR/J
MS	Multiple sclerosis
p.i.	post-infection
p.f.u.	plaque forming unit
TMEV	Theiler's murine encephalomyelitis virus
TMEV-DA	TMEV Daniels strain
TMEV-IDD	TMEV-induced demyelinating disease
$^{\text{TMEV}}$ SJL/JRj	TMEV infected SJL/JRj
$^{\text{TMEV}}$ SWR/J	TMEV infected SWR/J

Author contributions AV and DD initiated the project; AV designed the study; AV, ID, AT and LD performed the laboratorywork; AV analysed the results; AV wrote the original draft; AV, DD and JB reviewed the draft, JB acquired the fundings. All authors read and approved the final manuscript.

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Data availability All datasets, on which the conclusions of the manuscript rely on, are presented in the paper.

Declarations

Competing interests The authors declare no competing interests.

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