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Potential implication of new torque teno mini viruses in parapneumonic empyema in children

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ABSTRACT

An unexplained increasing incidence of parapneumonic empyema (PPE) in pneumonia cases has been reported in recent years. The present study investigated the genetic and biological specifications of new isolates of Torque Teno Mini Virus (TTMV) detected in pleural effusion (PE) samples from children hospitalised for severe pneumonia with PPE.

A pathogen discovery protocol was applied in undiagnosed PE samples and led us to the identification of 3 new isolates of TTMV (TTMV-LY). Isolated TTMV-LY genomes were transfected into A549 and HEK293T cells and viral replication was assessed by qPCR and full-length genome amplification. A549 cells were further infected with released TTMV-LY virions and the induced-innate immune response was measured by multiplex immunoassays.

Genetic analyses of the 3 TTMV-LY genomes revealed a classic genomic organisation but a weak identity (<64%) with known sequences. We demonstrated here the *in vitro* replication of TTMV-LY in alveolar epithelial cells and the effective release of infectious viral particles. We also showed a selective production of inflammatory mediators in response to TTMV infection.

This study reports the description of replicative TTMV-LY isolated from parapneumonic effusions of children hospitalised with PPE, suggesting the potential role of the virus in the pathogenesis of pneumonia.

INTRODUCTION

About 1.8 million children under the age of five years die from pneumonia every year [1]. A wide range of pathogens are recognised as causative agents of pneumonia, but 14-23% of cases remain etiologically undiagnosed despite continuing optimisation of diagnostic tests for the detection of respiratory pathogens [2,3].

Different methods have been developed for the identification of unsuspected, new, and divergent pathogens. In recent years, these techniques have led to the discovery of several viruses in respiratory specimens, including human metapneumovirus, bocavirus, WU and KI polyomaviruses, and Torque Teno Virus (TTV) [4]. Some of these have been subsequently confirmed as causative agents of lower respiratory tract infections (LRTI), but the involvement of most of these viruses remains a widely debated issue. TTV belongs to the recently created viral family *Anelloviridae,* composed of single-stranded circular DNA viruses, such as TTV (*Alphatorquevirus*), Torque Teno Midi Virus (*Gammatorquevirus*), and Torque Teno Mini Virus (TTMV - *Betatorquevirus*) [5].

In this study, we isolated and characterised 3 new species of TTMV (TTMV-LY) detected in pleural fluids from children hospitalised in France with parapneumonic empyema (PPE) by a method of pathogen discovery. This pathology, with an increasing incidence, is associated with a high morbidity and frequently requires prolonged hospitalisation and invasive procedures [6]. As the respiratory tract is thought to be a site of primary infection and continual replication of TTV [7], we conducted further investigations to evaluate to what extent these new viruses may be associated with the pathology. We report here that TTMV-LY isolates actively replicated in alveolar epithelial cells and were able to modulate their innate immune response.

EXPERIMENTAL PROCEDURES

Patients and Samples

A prospective study of 28 children, aged from 5 months to 14 years old (12 males and 16 females), hospitalised between April 4, 2007 and March 30, 2009 in the paediatric intensive care unit of Hospices Civils de Lyon for PPE was performed. This study was approved by all participating institutional review boards (Register: CE 06-139) and signed informed consent was obtained from all parents. Twenty-eight nasopharyngeal aspirates (NA) were obtained using disposable mucus extractors within 48h of admission. Twenty-five pleural effusion (PE) samples were collected at the time of chest drain insertion. The samples were kept frozen at -70°C until further study.

Respiratory pathogens nucleic acids detection

The presence of the 25 most common respiratory pathogens was tested in the 28 NA and 25 PE samples using the FTD Respiratory Pathogens 21+ assay (Fast Track Diagnostics, Luxembourg) in combination with the AgPath-ID One-Step RT-PCR Kit (Ambion, USA) after total nucleic acid extraction using the NucliSENS® easyMAG platform (bioMérieux, France, named easyMAG below) as recommended by the manufacturer.

TTMV isolation by pathogen discovery and genetic characterisation

Five negative PE samples were subjected to a pathogen discovery protocol adapted from Allander et al. [8] and based on the cloning and sequencing of random RT-PCR products. Homebrew sequence analysis software was used to identify new or divergent sequences from the one deposited in databases. Three out of five samples harbouring fragments of TTMV sequences were subjected to TTMV full-length genome amplification by inverse PCR with back-to-back TTMV-LY-9F and TTMV-LY-8R primers (Table 1). Amplicons were cloned and sequenced through both strands. Using the MEGA5 program, multiple alignments were generated by ClustalW, and phylogenetic trees were constructed by the neighbour-joining method. Predictive secondary structures were modelled with RNA structure 4.5 software. Refer to online supplementary materials for details of the procedures. The 3 full-length genomes obtained in the present study were deposited in Genbank under the following accession numbers: JX134044, JX134045 and JX134046.

TABLE 1. Primers and probes used in this study.

Primers[a]	Sequence	Nucleotide position	Target	GATC-site position	Amplification conditions
Inverse PCR					
TTMV-LY-9F	5'-GACCACAAACCGTCACTTAGTTCC-3'	42-65	*Betatorquevirus*	N/A	95°C for 1min 40 cycles: 95°C for 30sec 54°C for 1min 72°C for 3min 72°C for 10min
TTMV-LY-8R	5'-GCAATTTAAATTAGGTGGTTTTCCTG-3'	40-15			
TTMV-LY-11F	5'-CTAGRTCAGTCTGGCGGAACGG-3'	288-309	*Betatorquevirus*	N/A	
TTMV-LY-10R	5'-CCCCTTGACTACGGTGGTTTCAC-3'	278-256			
Real-time PCR					
TTMV-LY-F	5'-ATTCGAATGGCTGAGTTTATGC-3'	170-191	*Betatorquevirus*	210; 579; 2674	95°C for 3min 35 cycles: 95°C for 10sec 54°C for 30sec 72°C for 34sec
TTMV-LY-R	5'-CCTTGACTACGGTGGTTTCAC-3'	276-256			
TTMV-LY-P	5'-CCAGACGGAGACCGGATCACTTCA-3' (FAM-BHQ1)	194-217			
TTMV-LY-2F	5'-CACGAATTAGCCAAGACTGGGCAC-3'	2398-2421[b]	TTMV_LY2	512; 1382; 2456; 2732; 2746[b]	
TTMV-LY-2R	5'-TGCAGGCATTCGAGGGCTTGTT-3'	2494-2473[b]			
TTMV-LY-2P	5'-ACAGAGCCAGATCGACAGCAACTGCT-3' (FAM-BHQ1)	2445-2470[b]			

Nucleotide position and GATC- sites are indicated based on TGP96 (AB041962) sequence. N/A, Not Applicable; a- F, forward; R, reverse; P, probe; b- Nucleotide positions are based on TTMV-LY2 sequence

Cell culture and TTMV transfection or infection

Two cell lines were used to study the replication of these new isolates of TTMV: the human embryonic kidney 293T (HEK293T) cell line and A549 alveolar epithelial cell line. HEK293T were maintained in DMEM, 10% foetal bovine serum, 2mM glutamine and antibiotics, while A549 were maintained in F12K medium, 10% foetal bovine serum, 2mM glutamine and antibiotics. Cloned TTMV DNA was linearised with *Eco*RV and *Bam*HI (Roche Diagnostics, France). Viral and vector DNAs were co-transfected into cells (1 µg per 10^5 cells on 12-well plates) with JetPEI reagent (PolyPlus-transfection, France) as recommended by the manufacturer. Controls included transfection with vector alone or cells transfected with JetPEI alone. Transfected cultures were incubated overnight at 37°C, 5% CO_2. After 18h, cells were washed three times with PBS before adding fresh medium. The transfection efficiencies were optimised with a reporter plasmid. For the infection studies, healthy cells, seeded in 24-well plates, were infected with the supernatants of TTMV DNA-transfected cells that had been harvested 3 days post-transfection, and incubated at 37°C, 5% CO_2 for 2h, 7h, and 24h. Cells were

then washed 3 times with PBS and incubated with fresh medium for 6 hours. All experiments were performed at least twice, in duplicate.

TTMV-LY replication

For all experiments, supernatants were collected at each prescribed time, centrifuged, and immediately treated with DNaseI for 2h at 37°C and frozen at -80°C. Replicated TTMV DNA was extracted using easyMAG and eluted in 50µl. Cells were harvested in 2ml of NucliSENS lysis buffer (bioMérieux, France) for extraction of nucleic acids with easyMAG and eluted in 100µl of elution buffer. Total cellular DNA was then digested with *Dpn*I or *Nde*II, which respectively cut the dam-methylated GATC-sites of input TTMV and the non-methylated GATC-sites of replicative TTVMV (Table 1). To completely digest DNA and reduce the background level that is observed in qPCR, exonuclease III (ExoIII, Fermentas, Canada) was added as described elsewhere [9]. Full-length genomes of TTMV were detected by inverse PCR. Briefly, the reaction mix was composed of 5µl of nucleic acids, 1 µM of each primer TTMV-LY-11F and TTMV-LY-10R (Table 1), 2 mM of dNTP mix, and Expand High Fidelity enzyme mix (Invitrogen, Germany). Quantification of replicated TTMV was achieved by Taqman real-time PCR assays with primers targeting specifically a GATC-site. The qPCR assays were optimised as already recommended [10]. The amplification step was realised in 25 µl volumes containing 12.5 µl iQ Supermix (Biorad, USA), 0.3 µM each primers and probe (Table 1) and 5 µl of DNA. Amplification was detected with a CFX96 detection machine (Biorad, USA). DNA from untransfected cells was included in each reaction. For transfection studies, the replication rate was calculated as the ratio of the quantified amount of replicative TTMV detected after *DpnI* digestion to the total quantified amount of TTMV (replicative and residual input TTMV genomes).

Measurements of cytokine/chemokine release

Cell-free supernatants collected from the TTMV-infected A549 cell line cultures were assayed for simultaneous quantification of human EGF, Eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, VEGF by using a commercial multiplex bead-based immunoassay kit (Milliplex ; Millipore, USA).

RESULTS

Prevalence of respiratory pathogens in NA and PE positive samples

The identification of the main respiratory agents in NA and PE samples of children hospitalised for severe pneumonia was performed using a sensitive multiplex real-time molecular assay. In NA samples, rhinovirus, parainfluenza viruses, and human metapneumovirus were found as the main respiratory pathogens (Table 2). Bacterial agents were found in 80% of the PE samples whereas, 20% remained undiagnosed.

TABLE 2. Prevalence of respiratory pathogens in nasopharyngeal aspirates and pleural effusion samples from 28 children hospitalised with PPE.

	Nasopharyngeal aspirates (n=28)	**Pleural effusion (n=25)**
Bacterial detection		
Streptococcus pneumoniae	15	19
Staphylococcus aureus	2	1
Viral detection		
Rhinovirus	8	
Bocavirus	3	
Parainfluenza viruses	4	
Human Metapneumovirus	4	
Human coronavirus NL63	2	
Respiratory Syncitial Virus	2	
Influenza A and B	0	
Adenovirus	1	
Cases of co-detection	6	0
Undiagnosed	5 (18%)	5 (20%)
TTMV detection *		
Positive samples	8	9
Cases of co-detection	6 (with one or several viruses)	6 (with *S. pneumoniae*)

Data are presented as n or n (%).Clinical criteria were based on respiratory complaints and fever, difficulty in breathing, pulmonary infiltrates compatible with pneumonia, chest radiograph at admission confirmed by ultrasounds, white blood cell counts > 20 G/l or neutrophilis > 10 G/l and CRP level > 60 mg/l after 12 hours of fever. Undiagnosed NA and PE samples came from different patients, except for one of them Isolation and genetic characterisation of TTMV sequences in undiagnosed PE samples. *TTMV was detected by real-time PCR with primers and probes indicated in Table 1.

A pathogen discovery protocol based on random RT-PCR was performed on the 5 negative PE samples, and several short sequences related to TTMV were obtained for 3 of them. The characterisation of TTMV required the amplification and sequencing of the full-length virus genome. *Betatorquevirus*-specific inverse PCR was performed and the corresponding TTMV-LY genomes were amplified. The TTMV-LY genomes were composed of 2912 bp, 2979 bp, and 2915 bp for TTMV-LY1, -LY2, and -LY3, respectively. By PCR, the TTMV-LY genomes found in PE samples were also identified in the corresponding NA sample for each patient. Among the 3 patients for whom the PE samples were only positive for TTMV, 2 were male and 1 female. They were aged of 48, 7 and 37 months old respectively for TTMV-LY1, -LY2 and –LY3 and presented the same clinical features as the other patients of the cohort. Phylogenetic analyses were carried out on the nucleotide sequence of predictive ORF1, as recommended by the International Committee on Taxonomy of Viruses [5]. Based on the phylogenetic tree (Figure 1), all 3 new isolates of TTMV-LY belong to the genogroup I and are related to previously identified TGP96 (64% of maximum identity) and Pt-TTV8-II (44% of maximum identity) isolates. TTMV-LY isolates possess at least 3 ORFs: one major ORF1 composed of 673, 667, and 659 aa for TTMV-LY1, -LY2, and -LY3 respectively, and several overlapping ORFs. The TTMV-LY1 and -LY3 have 2 overlapping ORFs of 91 and 101 aa, and 97 and 129 aa, respectively, as opposed to TTMV-LY2, which has ORFs of 100 and 109 aa, and an additional ORF3 of 113 aa. One other feature of these viruses is the highly conserved 5'-untranslated region (UTR), as shown in Figure 2. Figure 2a shows that TTMV-LY2 possesses a significant insertion in this 5'-UTR, forming a predictive secondary structure of RNA sequence (Figure 2b). This structure appeared to be capable of forming a stable loop and dimer structure.

Transfection of the TTMV-LY genomes into cells and i*n vitro* replication

After transfection of the linearised virus genomes, we monitored i) the replication of the new TTMV isolates in the A549 and HEK293T cells, and ii) the release of viral particles in the supernatants, by quantifying newly synthesised genomes with a specific normalised qPCR assay for one week. We initially observed that, as shown in Figure 3A, all the TTMV-LY isolates actively replicated in both cell lines. There was no significant difference between the replication mean rates for the 3 isolates in A549 cells (25%), and in HEK293T (10%). As observed in the kinetic experiments (Figure 3b), the mean rate of replication rose regularly from day 2 to day 4, reaching 35% of the replicative form of TTMV-LY in both cell lines. This replication rate remained relatively constant in A549 cells until day 7, but in

HEK293T cells it slightly decreased beginning day 4. The release of TTMV-LY particles showed a similar pattern as to the replication rate within the cells, with a peak at day 4 post-transfection (p.t.), with an 8-fold increase for A549 and a 20-fold one for HEK293T supernatants.

Furthermore, we ensured that the replicative TTMV-LY detected in Figure 3 effectively corresponded to viral particles and not merely to partial fragments of DNA. For this purpose, the complete genome amplification of TTMV-LY in the A549 cells (Figure 4a) and supernatants (Figure 4b) were measured. We observed the presence of a 2.9 kb PCR fragment representing the recircularised replicative TTMV for the 3 TTMV-LY isolates, verified by sequencing analyses (data not shown). We confirmed that circular full-length genomes were present in cells and were released into the supernatants of A549 cells, indicating the presence of viral particles.

TTMV-LY infectious particles replicate in alveolar epithelial cells.

The capacity of producing replicative particles was measured by quantifying the TTMV-LY genomes in A549 cells. We exposed pulmonary cells for 2h, 7h, and 24h with the neosynthetised virus particles released at day 3 p.t. into the supernatants of the transfected A549 and HEK293T cells. We noticed that the optimal condition for A549-infected TTMV-LY was after an incubation of 2h, and 7h, with the supernatants of A549 and HEK293T transfected cells, respectively (Figures 5a and 5b). We also observed that the efficiency of TTMV A549 cell entry was more significant when the viral load was the highest (10^7 genome equivalents). Moreover, the quantity of TTMV-LY within the A549 cells was systematically greater for infections with the supernatants of A549 cells than within HEK293T cells, with $1.7x10^6$ versus $5.6x10^4$ after 2h of infection with 10^7 genome equivalents, suggesting that the viral particles produced by pulmonary cells may be more infectious. This result was confirmed by the quantification of replicated TTMV-LY after infection of the 2 cell lines (Figures 5c). Infected A549 cells systematically showed the highest viral titre with 48- and 167-fold increases for the infections with the HEK293T and A549 supernatants, respectively, versus 22- and 44-fold increases for the HEK293T cells at day 3. This observation was made for all 3 TTMV-LY (data not shown).

Each individual TTMV-LY modulates the innate immune response of pulmonary epithelial cells in a specific manner.

In this study, we used a 29-plex bead-based immunoassay to measure the levels of a wide range of inflammatory mediators in the supernatant of pulmonary cells infected with -LY1, -LY2 or TTMV-LY3.

As shown in Table 3, at 6h, post-infection TTMV-LY induced the production of several cytokines and chemokines, which are significantly enhanced compared to the ones produced in uninfected cells. We observed that TTMV-LY1 drives the production of a large number of pro-inflammatory (IFNγ, RANTES, IL-2, IL-12), chemotactic (MIP-1b), and anti-inflammatory cytokines (IL-10, IL-13), as well as growth factors (G-CSF, GM-CSF, IL-7). The 2 other TTMV-LY induce a weaker response with the induction of RANTES, IL-12(p40), MCP-1, VEGF, and IL-13 for TTMV-LY2 and IL-2, IL-12(p40) and MCP-1 for TTMV-LY3. Only IL-12 was released into supernatants for the 3 TTMV-LY after infection of the cells.

TABLE 3. Secretion of inflammatory mediators into the supernatant of 2h-infected A549 cells at 6h post-infection.

	Soluble Mediators	Mock	TTMV-LY1	TTMV-LY2	TTMV-LY3
Pro-inflammatory cytokines	IFN-γ	4.5 ± 3.0	**20.5 ± 4.9**	7.8 ± 2.7	7.2 ± 0.8
	IL-2	26.6 ± 5.2	**51.3 ± 6.8**	32.3 ± 2.4	**33.4 ± 1.2**[#]
	IL-12(p40)	16.6 ± 4.2	**36.3 ± 4.9**[#]	**22.2 ± 1.0**[#]	**22.3 ± 1.1**[#]
	IL-12(p70)	10.2 ± 1.8	**20.9 ± 2.1**[#]	10.1 ± 0.8	10.6 ± 0.6
Anti-inflammatory cytokines	IL-10	54.8 ± 4.9	**95.7 ± 5.4**[#]	60.2 ± 1.2	55.3 ± 0.9
	IL-13	82.0 ± 6.1	**120.2 ± 1.3**[#]	**94.6 ± 1.2**[#]	89.2 ± 3.8
Chemotactic cytokines	RANTES	28.1 ± 0.1	**67.7 ± 12.4**[#]	**71.6 ± 14.2**[#]	33.1 ± 2.4
	MIP-1β	43.4 ± 1.9	**67.9 ± 0.4**[¶]	43.5 ± 2.7	44.2 ± 2.6
	MCP-1	868 ± 197	1271 ± 48	**2108 ± 524**	1610 ± 550
Growth factors	G-CSF	55.5 ± 3.6	**176.2 ± 38.1**[#]	58.1 ± 1.7	58.5 ± 4.0
	GM-CSF	30.9 ± 7.5	**56.9 ± 1.0**[#]	44.1 ± 2.7	40.0 ± 5.9
	IL-7	37.8 ± 6.5	**77.8 ± 7.1**[#]	41.1 ± 1.1	39.2 ± 1.3
	VEGF	156.5 ± 20.0	218.6 ± 13.5	**254.6 ± 32.8**[#]	220.9 ± 42.3

Data are expressed as mean pg/ml ± SD. Experiments were performed twice in duplicate.

[#] Statistically different from mock at $p<0.05$ (Student's t test).

[¶] Statistically different from mock at $p<0.01$ (Student's t test).

DISCUSSION

This study examined the potential involvement of new isolates of Torque Teno Mini Virus, identified in pleural effusion samples, in the pathogenesis of severe pneumonia in children. The main findings are that i) TTMV-LY can deeply colonise lungs, ii) alveolar epithelial cells, where efficient replication occurred, are permissive to the TTMV-LY, iii) TTMV-LY infection modulates the innate immune response of pulmonary cells by inducing the production of inflammatory mediators.

Parapneumonic empyemas result from an inflammation of the pleura space as a consequence of infection by bacterial pathogens [11]. Bacteria are frequently described in pleural fluids, also confirmed in this study in PE samples, but the presence of a viral origin remains poorly documented [12,13]. This study is the first to reveal the presence of an anellovirus in parapneumonic effusions. Anelloviruses are characterised by extreme genetic diversity, a high prevalence in various populations, and a wide distribution in body fluids. This ubiquity, together with the absence of suitable *in vitro* culture systems, has hampered progress in the investigation of this group of viruses [14].

In previous molecular epidemiological studies, unequivocal demonstration that anelloviruses are implicated in the pathogenesis and severity of disease is still lacking or is poorly demonstrated [15,16]. Here, we found the presence of full-length TTMV genome in NA and PE in children hospitalised with severe pneumonia. In this cohort as in a Chinese cohort of 216 NA samples of children suffering from LRTI (data not shown), no correlation has been observed between the severity of the disease and the presence of TTMV, the age or the sex of the patients. However, the presence of TTMV complete genome linked to the viral replication may indicate its role in the pathogenesis of pneumonia.

This study reports the isolation of 3 new full-length TTMV genomes (TTMV-LY) in children with severe pneumonia. As a first step, the biological properties of these new isolates of TTMV-LY were investigated. We first transfected the full-length genome of the three TTMV-LY viruses to ascertain whether the viral replication takes place in HEK293T cells, as already demonstrated [17,18], and subsequently in alveolar epithelial A549 cells that act as a model of type II pneumocytes [19]. We described for the first time that TTMV, like TTV, was actually able to replicate in cell lines after transfection of its genome and lead to the production of infectious virions. We observed that the viral infection was more efficient in alveolar cells than in kidney cells, suggesting a better tropism of the TTMV-LY in pulmonary cells and a strengthened role in the pathogenesis of LRTI. We hypothesised

that, like porcine circoviruses and papillomaviruses, the potential pathogenicity of TTMV could be restricted to some species or a group of species [20,21]. Indeed, in this study, high genetic TTMV diversity was observed only among different patients, but the same full-length genome was systematically found in the different samples taken from the same patient. All 3 genomes, moreover, belonged to the same phylogenetic branch of *Betatorquevirus* (Group I). We investigated the conserved 5'UTR genome sequence of the 3 TTMV-LY genome sequences to determine if specific replication patterns were present. No assumed origin of replication (_AGT_TTACA) was identified but an insertion of 200 bases forming a stem-loop structure in the conserved 5'-UTR was detected in the TTMV-LY2. As previously described, this secondary structure could be involved in the viral pathogenicity as enhancer and promoter elements [22]. Despite that TTMV-LY2 presents this secondary structure, no gain in the replication yield was observed compared to the other TTMV-LY. However the effects of such genetic modifications could be expressed in another biological manner, in response to the interaction with the host cell, as observed for many viruses.

As a second step, we investigated the response of the host A549 cells replying to TTMV-LY infection by measuring the production of soluble mediators implicated in the inflammation process. Alveolar type II epithelial cells maintain alveolar integrity by forming the alveolar barrier, producing surfactants and repairing injured type I epithelium [23]. Moreover they are an integral part of the lung innate immunity, acting to intensify the function of dendritic cells and alveolar macrophages by cytokines secretion [24,25]. No cytopathic effect was observed in A549 cells, but infection with TTMV-LY induced a complex innate immune response, differently modulated by the 3 isolates. Thus, TTMV-LY infection resulted in the production of not only pro-inflammatory cytokines, including IFNγ as previously described [26], but also significant levels of IL-10 or IL-13 anti-inflammatory cytokines were measured, corroborating that TTMV-LY may modify innate immune balance, as already demonstrated for TTV [27]. The fact that different isolates of TTMV can impact differently on the production of soluble mediators is not surprising in view of what has been observed with other viral infections [28,29], and may reflect that they are recognised by different receptors and/or interact through different molecular pathways. IL-12 seems to play a central role in the response to TTMV-LY infection. This cytokine, enhancing the proliferation and cytotoxicity of activated T and natural killer cells, is thought to be a key factor in the regulation of the host defence against many intracellular pathogens [30]. We may assume

that TTMV-LY-mediated lung inflammation may result from the abundant secretion of IL-12, although further studies would be needed to prove this.

Taken together, these results suggest that TTMV-LY isolates can invade the alveolar cells where they replicate, and induce innate immune imbalance in the respiratory tract. Further studies aimed at understanding the physiopathological implications of TTMV as etiological agent in respiratory diseases are necessary, as required for every newly discovered pathogen. These investigations would allow to learn about the presumed need to enlarge the range of researched pathogens to the anelloviruses in case of parapneumonic empyema, in order to improve the diagnosis efficiency and treatment of this pathology.

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FIGURE LEGENDS

Figure 1. Phylogenetic tree of the ORF1 nucleotide sequence of TTMV-LY1, -LY2, -LY3 and *Betatorquevirus* constructed by the neighbour-joining method. TTMV-LY genomes isolated from PE samples are circled. The scale bar represents the number of nucleotide substitutions per position.

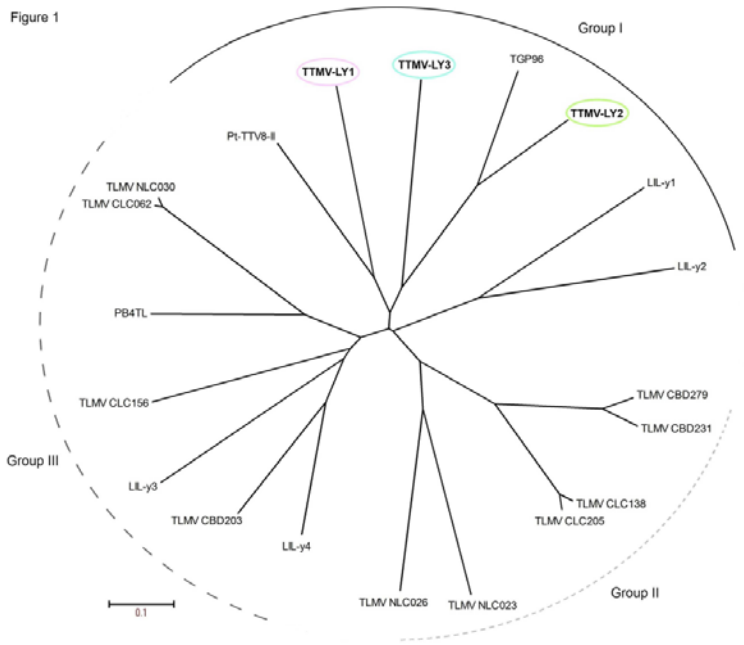


Figure 2. Multiple alignment in the 5'-UTR of the newly defined phylogenetic group I nucleotide sequences revealed the presence of a significant insertion in the TTMV-LY2 genome (a) which could form a stem-loop pattern in the predictive secondary structure (b). Residues that are identical among the sequences are given a black background. Lines between residues indicate deletions between the sequences.

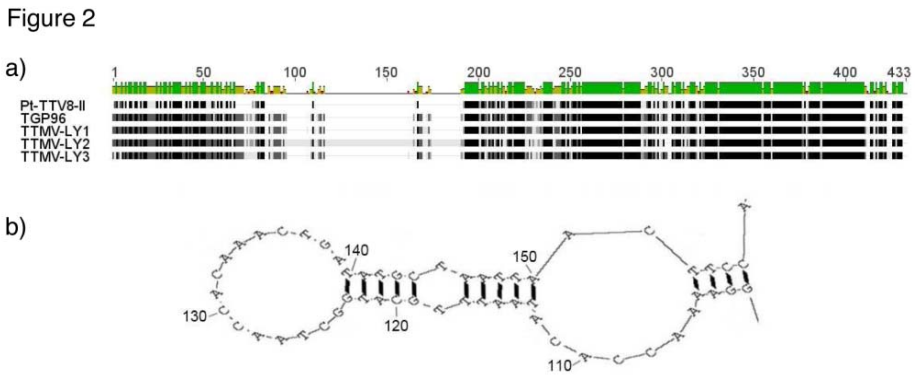


Figure 3. Replication of the TTMV-LY genomes after transfection produce more replicative TTMV in HEK293T cells than in A549 cells as quantified by real-time qPCR.

Histograms represent the mean rate of replication of the TTMV-LY in the cells transfected with the linearised full-length genomes. Replication of TTMV-LY was (a) measured at day 3 post-transfection (p.t.), or (b) monitored during a week following transfection. In b) curves indicate the quantification of TTMV-LY1 in the culture medium of cells at days 0 to 7 p.t. after DNaseI treatment and nucleic acid extraction. Results are expressed as the ratio of quantified TTMV-LY1 at day 1 to 7 p.t. to quantified TTMV-LY1 at day 0. Data are shown only for TTMV-LY1, because the replication profile among the 3 viruses is similar. Standard deviations between experiments are shown.

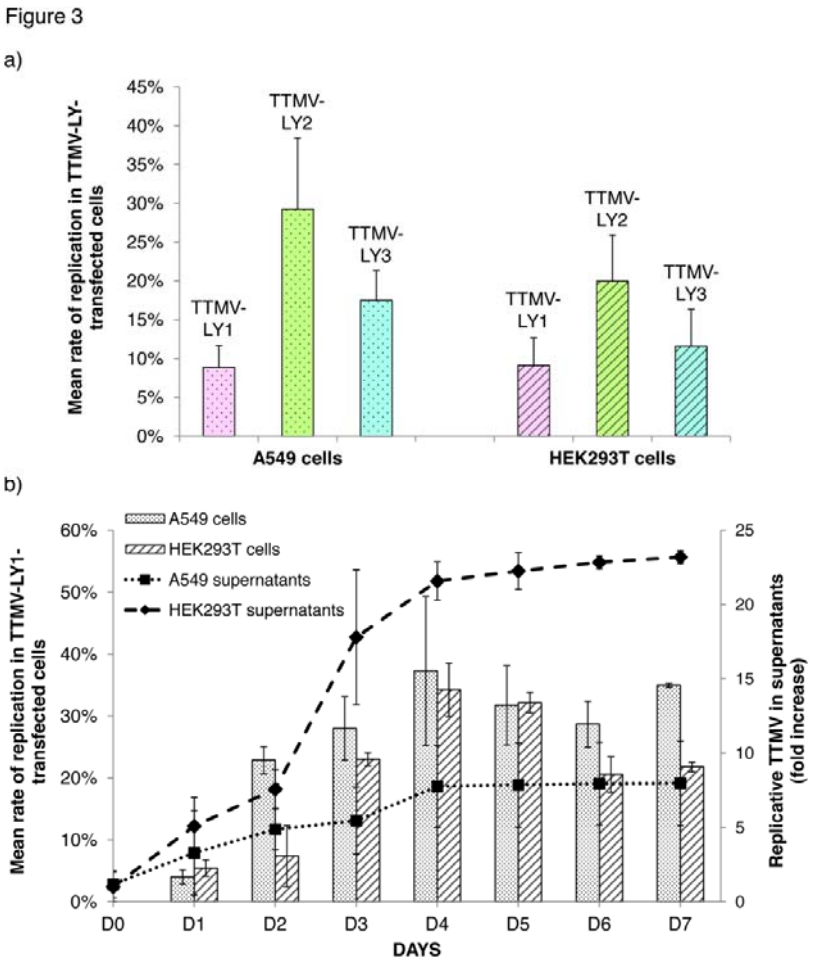


Figure 4. Detection, by PCR with back-to-back primers, of replicative recircularised genomes of TTMV-LY in the A549 cells (a) and in the supernatants of cells (b) at day 3 post-transfection.

LY1, LY2, and LY3: cells transfected with the TTMV-LY1, -LY2, and -LY3 genomes, respectively. NC1, NC2, and NC3: negative control for each genome, respectively. UC: Uninfected cells. M: DNA molecular weight marker.

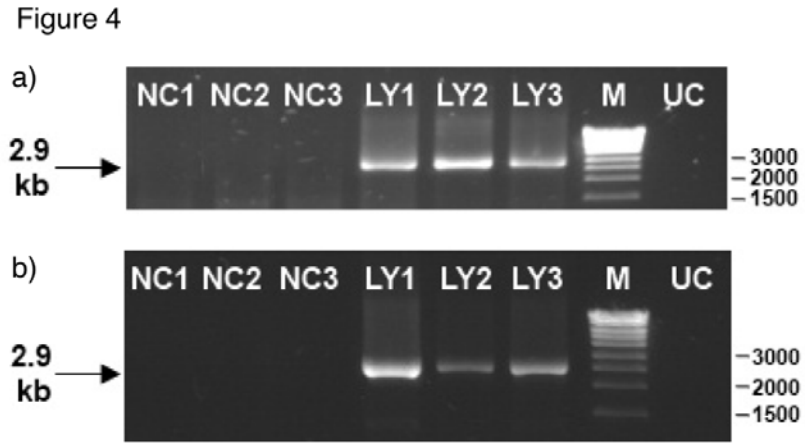
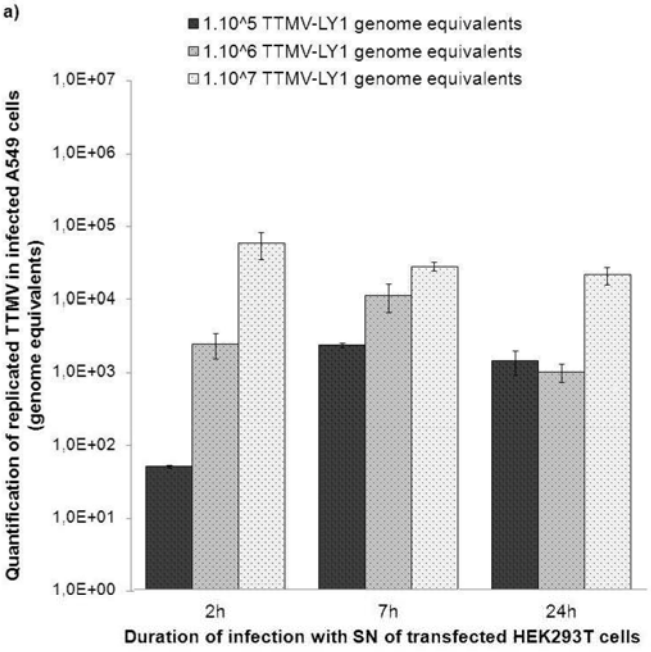


Figure 5. TTMV-LY isolates infect preferentially the A549 alveolar cell line than the HEK293T cell line, as quantified by qPCR.

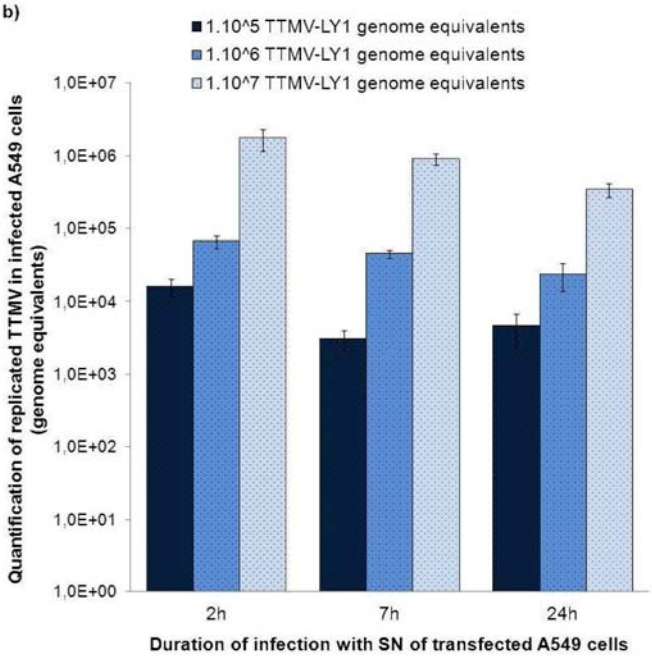
For a) and b), A549 cells were incubated with TTMV-LY1 for 2h, 7h, and 24h with 3 different viral loads of 10^5, 10^6, and 10^7 genome equivalents, harvested from the supernatants (SN) of transfected A549 or HEK293T cells at day 3 p.t. Standard deviations between replicates are shown. The quantity of TTMV-LY measured in culture-medium-exposed cells was null.

For c), the quantity of replicated TTMV-LY was measured at day 0 and day 3 post-infection in the 2h-exposed A549 and HEK293T cells with $1x10^7$ genome equivalents viral load. Results are expressed as fold increase compared to cells exposed with the supernatants of cells exposed to the TTMV-LY genomes without transfection reagent.

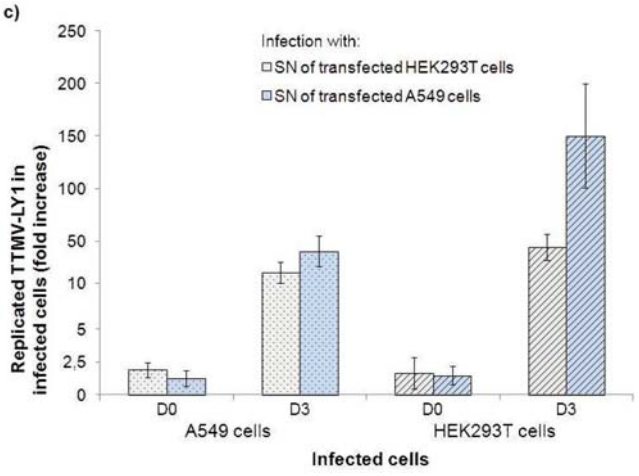
a)


1.10^5 TTMV-LY1 genome equivalents
1.10^6 TTMV-LY1 genome equivalents
1.10^7 TTMV-LY1 genome equivalents
Quantification of replicated TTMV in infected A549 cells (genome equivalents)
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
1,0E+00
2h
7h
24h
Duration of infection with SN of transfected HEK293T cells

b)


1.10^5 TTMV-LY1 genome equivalents
1.10^6 TTMV-LY1 genome equivalents
1.10^7 TTMV-LY1 genome equivalents
Quantification of replicated TTMV in infected A549 cells (genome equivalents)
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
1,0E+00
2h
7h
24h
Duration of infection with SN of transfected A549 cells

c)


Infection with:
SN of transfected HEK293T cells
SN of transfected A549 cells
Replicated TTMV-LY1 in infected cells (fold increase)
250
200
150
100
50
10
5
2,5
0
D0
D3
D0
D3
A549 cells
HEK293T cells
Infected cells