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Torque Teno Virus in Nasopharyngeal Aspirate of Children With Viral Respiratory Infections

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Background: Torque teno virus (TTV) is a ubiquitous anellovirus responsible for persistent infections and is considered a marker of immune function. The role of TTV as a facilitator of respiratory infections (RIs) is unknown.
Objectives: Our aim was to estimate, in a prospective study, the prevalence of TTV in the nasopharyngeal aspirate (NPA) of hospitalized children <5 years old, with RIs and correlate them with outcomes and immune response.
Patients and Methods: NPA was taken for testing of 16 respiratory viruses by reverse transcription-polymerase chain reaction (PCR), TTV PCR, and immunologic study.
Results: Sixty hospitalized children with an RI were included. A total of 51/60 patients had positive common respiratory viral (CRV) identification. A total of 23/60 (38.3%) children were TTV+ in NPA. TTV+ patients had other CRVs in 100% of cases versus 78.3% in TTV− ($P = 0.029$). The TTV+ patients tended to be older, have fever, and to need pediatric intensive care unit admission more often than TTV− patients. Abnormal chest radiograph was more frequent in the TTV+ patients, odds ratios 2.6 (95% CI: 1.3–5.2). The genetic expression of filaggrin (involved in epithelial barrier integrity) was lower in TTV+ patients; however, the levels of filaggrin in the NPA were increased.
Conclusions: TTV infection is common in children with RI and could be associated with abnormal imaging in radiograph, greater severity and an alteration in filaggrin gene expression and protein release.

Key Words: Torque teno virus, infants, viral respiratory infections

(*Pediatr Infect Dis J* 2023;42:184–188)

INTRODUCTION

Torque teno virus (TTV) is a prototype anellovirus, a small ubiquitous DNA virus responsible for persistent asymptomatic infections.[1,2] TTV is a marker of immunologic status.[3] An increase in TTV replication has been observed in sepsis,[4] HIV infection,[5–7] untreated cancer,[8] bone marrow transplantation[9,10] and solid organ transplantation[11–13] as a potential endogenous marker of immunosuppression. In terms of respiratory infections (RIs), TTV has been found in children with recurrent types of pneumonia, showing its potential to infect the respiratory tract.[14] It has also been associated with bronchopneumonia, and its role in asthma is under study.[15,16] As far as we know, there have been few studies of TTV in respiratory samples of children with respiratory tract infections, correlating its presence with the immunologic state or its clinical evolution.[15] The role of TTV as a facilitator of RIs or airway inflammation remains to be determined.

The objective of this study was to determine the prevalence of TTV in hospitalized children with RIs, analyzing their nasopharyngeal aspirate (NPA) samples and how viral detection correlates with individual clinical evolution. We also analyzed the innate immune response of the children who were positive for TTV.

PATIENTS AND METHODS

This is a substudy of an ongoing prospective investigation of respiratory tract infections in children, approved by the Medical Ethics Committee of Carlos III Health Institute Ethics Committee of Hospital La Paz and the Ethics Committee of Hospital Severo Ochoa. All research was performed in accordance with regulations and the Declaration of Helsinki. Informed consent was obtained from all parents or legal guardians.

Clinical Assessment

This was a multicenter study performed in Madrid, Spain, between January and June 2021. The study population was comprised of children younger than 5 years of age with acute respiratory infection (ARI) admitted to either of the participating hospitals, Severo Ochoa (Leganés) or La Paz University Hospital (Madrid). The exclusion criterion was a refusal to participate in the study. Patients were evaluated by an attending physician, and the patients' clinical characteristics were analyzed. During their hospital stay, and as part of the study, a physician completed a questionnaire with the following variables: age; sex; month of admission; clinical diagnosis; history of prematurity or underlying chronic diseases; need for oxygen therapy, evaluated via transcutaneous oxygen saturation; fever; maximum axillary temperature; presence of infiltrates and/or atelectasis in chest radiographs; administration of antibiotic therapy; length of hospital stay; need for admission to a pediatric intensive care unit (PICU); total white blood cell count; serum C-reactive protein levels (mg/L) and blood culture results (in those cases for which such tests had been performed).

Acute expiratory wheezing was bronchiolitis when it occurred for the first time in children younger than 2 years of age,

Accepted for publication November 1, 2022
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Funded by projects PI18CIII/0009, PI18/00177, and PI21/00377, funded by Instituto de Salud Carlos III (ISCIII) and co-funded by the European Union.
There are no conflicts of interest.
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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (www.pidj.com).

ISSN: 0891-3668/23/4203-0184
DOI: 10.1097/INF.0000000000003796

following the classic criteria of McConnochie.[17] All other episodes of acute expiratory wheezing were recurrent wheezing. Laryngotracheobronchitis was associated with inspiratory stridor and wheezing. Laryngitis was associated with inspiratory stridor without wheezing. Cases with both focal infiltrate and consolidation in chest radiographs were, in the absence of wheezing, classified as pneumonia. However, those cases with wheezing were classified as bronchiolitis or recurrent wheezing as appropriate.

Viral Studies

Specimens consisted of NPAs that were obtained from each patient at admission. NPAs were sent for virological investigation to the Respiratory Viruses and Influenza Unit at the National Center for Microbiology (ISCIII), Madrid, Spain. Samples were stored at 4°C in a refrigerator and were processed within 24 hours after collection. Upon reception, 3 aliquots were prepared and stored at −80°C. Both the reception and the NPA sample processing areas are separated from those defined as working areas.

RNA and DNA from 200-µL aliquots of NPAs were extracted with the QIAamp Mini Elute Virus spin kit in an automated extractor (QIAcube, Qiagen, Valencia, CA).

Detection of respiratory viruses was performed by 4 independent multiplex reverse transcription-polymerase chain reaction (RT-PCR) assays. The first assay detected Influenza A, B and C viruses; the second was used to detect parainfluenza viruses 1–4, human rhinoviruses (HRVs), and enteroviruses; and the third assay detected the presence of respiratory syncytial virus (RSV) types A and B, human metapneumovirus, human bocavirus and human adenoviruses (HAdVs). These 3 assays were real-time multiplex RT-PCRs and used the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Waltham, Massachusetts, USA). A fourth multiplex RT-PCR was used for the investigation of human coronavirus (HCoV), using generic primers that were able to detect both alpha and beta coronavirus. Typing of HCoV was performed using a reverse-specific primer for detection of HCoV 229E, HCoV NL63, HCoV OC43 and HCoV HKU1. Primers and Taqman probes for the 3 independent multiplex real-time RT-PCRs were based on previously published designs by our group,[18] and the HCoV primers are available on request.

Torque Teno Virus Study

A generic PCR assay was designed to detect specific viruses from the Alphatorquevirus genus, due to this genus is clinically important and it is the most detected genus of torque teno viruses in human samples. Previous studies had described a variation in the rate of positive detection depending on the target site of amplification in the PCR. Given that the untranslated regions (UTRs) of the genome are more conserved as compared with the open reading frames, primers designed in the UTRs will cross-match many genotypes and increase the rate of detection.[19,20]

The screening primers designed ruled out the detection of Betatorquevirus, Gammatorquevirus genera and other environmental Anelloviruses. Forward primer U5F (5′-YKTCGTICACTTCCTGGGC-3′) and the reverse primer U5R (5′-CGAGCCCGAATTGCCCC-3′), were used at a 10-pmol concentration in the reaction mixture.

This method involved cycling consisting of a denaturation cycle of 95°C for 5 minutes, followed by 40 cycles with a denaturation step of 30 seconds at 95°C, an annealing step of 30 seconds at 57°C and an extension step of 1 minute at 72°C. The final extension step was 72°C for 5 minutes.

Amplified products (~180 bp) were visualized by 2% agarose gel electrophoresis containing 5 mg/mL of GreenGel in 1x Tris borate buffer.

Immunologic Study

A portion of each NPA sample was centrifuged to obtain the cellular pellet and supernatant. Samples with mucus were filtered with a 40-µm nylon filter. The pellet was resuspended in 0.7 mL Qiazol Lysis Reagent (Qiagen, Hilden, Germany) and frozen at −80°C. The supernatants were also frozen at −80°C.

We purified 500 ng of RNA from the NPA cell pellet with phenol-chloroform, where total mRNA was isolated from Qiazol by the use of chloroform and isopropanol precipitation, then dissolved in water and finally quantified by a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). It was then reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and analyzed by semi-quantitative real-time PCR on a StepOnePlus Real-Time PCR System with TaqMan probes for gene expression (Applied Biosystems) detection of 18s, *IFNG, TLR3, FLG*, AREG, interleukin (*IL*)-*13*, *IL-33*, *IL-10* and TaqMan Gene Expression MasterMix (Applied Biosystems), following the manufacturer's guidelines. Relative gene expression was calculated using the Cycle Threshold (Ct) and the $2^{-\Delta\Delta Ct}$ method,[21] where

$$\Delta\Delta Ct = \Delta Ct_{TTV+} - \Delta Ct_{TTV-} \text{ and } \Delta Ct = \Delta Ct_{gene} - \Delta Ct_{House\ keeping\ gene}$$

In the NPA supernatant, filaggrin was analyzed by an enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Cone Corp., TX), following the manufacturer's instructions.

Statistical Analysis

The descriptive data were expressed as mean and first and third quartile (interquartile range [IQR]) for the continuous variables, and through counts and percentages for the categorical variables.

The continuous variables that followed a normal distribution were compared using a one-way analysis of variance with Bonferroni correction, or through T tests. When the distribution was not normal, we used the Mann–Whitney U test or Kruskal–Wallis test with Dunn correction. The categorical variables were compared using a chi-squared test or Fisher exact test, and results were expressed as odds ratios (ORs). Receiver operating characteristic (ROC) curve was performed for biomarker determination, considering good AUC as over 0.7.

P values <0.05 were considered statistically significant, and CIs were calculated at 95% for all the estimations. The analyses were performed using SPSS software (version 21; SPSS Inc, Chicago, IL) and with Graph-Pad Prism 8 (GraphPad Software Inc., San Diego, CA).

RESULTS

The study population consisted of 60 hospitalized children with a diagnosis of RI. A total of 51/60 (85%) patients had a positive common respiratory viral (CRV) identification, and 24 (47%) were coinfected with more than one virus. The median age was 11.8 months (IQR 1.4–23.5), 66.7% were male, and 9 patients had been born preterm (14.3%). The children were mainly recruited (75%) in April and May of 2021, coinciding with this pandemic year with a higher incidence of RSV RIs during those months. All children were tested for SARS-CoV-2 and were negative. The most identified viruses were RSV (27/60; 45%) and HRV (26/60; 43.3%) in a similar proportion, followed by human bocavirus (14; 23.3%) and HAdV (9; 15%), 2 parainfluenzas, 1 HCoV NL63, and 1 metapneumovirus. TTV was identified in a total of 23/60 (38.3%) children.

Regarding clinical data, 28/60 (46.6%) children had fever, with a median maximum temperature of 38.5°C (IQR 38–39.1), and 44/60 (73.3%) had hypoxia, with a median duration of 3 days of oxygen supplementation (IQR 2–4). Eleven patients had infiltrated or atelectasis on radiograph (18%). Bronchiolitis was the most frequent diagnosis (29/60; 48.3%), followed by 22/60 (36.6%)

recurrent wheezing episodes. There were also 3 febrile syndromes, 3 upper RIs (1 with otitis), and 1 pneumonia, 1 laryngitis, and 1 laryngotracheobronchitis. The median hospital stay was 4 days (IQR 2–6.5), and 7/60 (11.6%) required PICU admission.

Comparison Between TTV-positive and -negative Patients

The 23 positive TTV patients were compared with the 37 TTV-negative children. The clinical data are shown in Table 1. Viral coinfection with another CRV was present in 100% (23/23) of the TTV-positive patients versus 78.3% (29/37) in the TTV-negative patients, $P = 0.029$. No specific CRV was significantly associated with the presence of TTV being RSV and HRV the most frequent in both groups (Table, Supplemental Digital Content 1, http://links.lww.com/INF/E896). The 2 positive parainfluenza cases were associated with the detection of TTV in NPA. Viral CRV coinfections were not associated with TTV presence.

The TTV-positive patients were slightly older and tended to have fever, and to need more frequent PICU admission than the negative ones, although the differences did not reach statistical significance. The TTV-positive children were also more likely to have radiologic abnormalities, OR 2.6 (95% CI: 1.3–5.2), $P = 0.030$.

Evaluation of the Immune Response and Barrier Integrity in Children With and Without TTV Infection

An aliquot of NPA was available in 22 patients, 11 with positive TTV and another 11 with negative TTV.

Interestingly, filaggrin mRNA (a gene involved in epithelial barrier integrity) was found more often expressed in TTV− infants compared with the TTV+ (44.4% versus 0%; $P = 0.03$; Fig. 1A). When we analyzed the filaggrin protein by ELISA in the NPA supernatants, we found increased levels of filaggrin in the TTV+ group (35.1 ± 2.1 ng/mL versus 26.3 ± 3.1 ng/mL; $P = 0.03$; Fig. 1B). The levels of filaggrin in the NPA supernatant were able to differentiate TTV+ versus TTV− samples by ROC curve (AUC = 0.76; 95% CI: 0.55–0.97; $P = 0.03$; Fig. 1C). When we evaluated other genes involved in the immune response (*TLR3*, *IL-33*, *IL-10*, *IFNG* and *IL-13*), we did not find significant differences between the TTV− and TTV+ ($P > 0.05$), although more TTV+ infants expressed *TLR3* and *IL33* compared with the TTV− (50% versus 22.2% and 50% versus 11.1%, respectively) (Fig. 1A).

DISCUSSION

The prevalence of TTV infection in children with ARI was high in our study, reaching 38.3% of cases. It was associated with infection by another respiratory virus in all cases. The children with detection of TTV in NPA had more often pneumonia on radiography, were slightly older, and tended to have a clinical course with more fever and admission to the PICU (near statistical significance). Our results suggest that TTV infection may be a marker of impaired immune response and a facilitator of RIs and greater severity. The study of our patients' immune response showed that TTV+ children have a higher abundance of the filaggrin protein in NPA, with a lower gene expression. This result could mean that there is an alteration of the epithelial barrier in the TTV+ group, which is associated with liberation of this molecule to the supernatant of their NPA. Filaggrin is a protein involved in the integrity of the epithelial barrier and could play a role in allowing or favoring these RIs.[22]

Chronic TTV infections have been reported in healthy individuals, and colonization is considered to begin very early in life, possibly transplacental in some cases or in the family environment by the fecal-oral route.[23,24] Colonization increases with age, as our results suggest with older age of infected infants. Although the prevalence of TTV in blood samples has been more often studied than in respiratory secretions, the respiratory route is a frequent path of dissemination and contagion.[15] The role of TTV in pediatric RIs is poorly understood. Maggi et al,[15] in the most important study in this regard, found that the presence of TTV was associated with bronchopneumonia and other respiratory viral infections, similar to our study. They hypothesized that coinfection could increase the severity of other RIs. Our study supports this hypothesis as we observed that our TTV+ coinfected patients suffered a more severe clinical outcome, with higher frequency of pneumonia and with a tendency to need more often PICU admission.

Studying the immune response in the respiratory secretions of these children is tempting, to better understand the

TABLE 1. Comparison between TTV-positive and TTV-negative children

Clinical Feature	TTV-positive (n = 23)	TTV-negative (n = 37)	OR (95% CI)	*P*
Male	14/23 (60.9%)	27/37 (73.0%)		0.582
Age (months)	12.2 (4.7–21.7)	11.8 (1.2–31.8)		**0.036**
Temperature ≥38°C	14/23 (50.8%)	14/37 (37.8%)		0.068
Highest temperature (IQR)	38.4 (37.9–38.9)	38.8 (38–39.4)		0.208
Hypoxia ($SatO_2$ < 93%)	19/23 (82.6%)	25/37 (67.5%)		0.156
Abnormal radiograph	**8/23 (34.8%)**	**3/37 (8.1%)**	**2.6 (1.3–5.2)**	**0.030**
Antibiotic treatment	6/23 (26%)	8/37 (21.6%)		0.479
Diagnosis				0.413
Wheezing episode	8/23 (34.7%)	14/37 (37.8%)		
Bronchiolitis	13/23 (56.5%)	16/37 (43.2%)		
Pneumonia	0	1 (2.7%)		
Blood test				
Leukocytes (cells/mm^3) (IQR)	10,500 (8080–16,750)	13,260 (8520–16,420)		0.649
C-reactive protein (mg/L) (IQR)	11 (3–22)	7.9 (3.6–51)		0.984
CRV in NPA	**23/23 (100%)**	**29/37 (78.3)**	**0.55 (0.43–0.71)***	**0.019**
Outcomes				
Hospital stay (days) (IQR)	5 (3–7)	3 (2–5)		0.948
Fever duration (days) (IQR)	2 (1–4)	2 (1–4)		0.856
Hypoxia duration (days) (IQR)	3 (2–5)	3 (2–4)		0.158
PICU admission	**5/23 (21.7%)**	**2/37 (5.4%)**		0.061
Stay >7 days	7/23 (30.4%)	8/37 (21.6%)		0.410

CRV indicates common respiratory viruses; IQR, interquartile range; NPA, nasopharyngeal aspirate; OR, odds ratio; $SatO_2$, oxygen saturation; TTV, torque teno virus.
*Negative TTV was a protective factor for CRV identification. Significant differences are in bold.

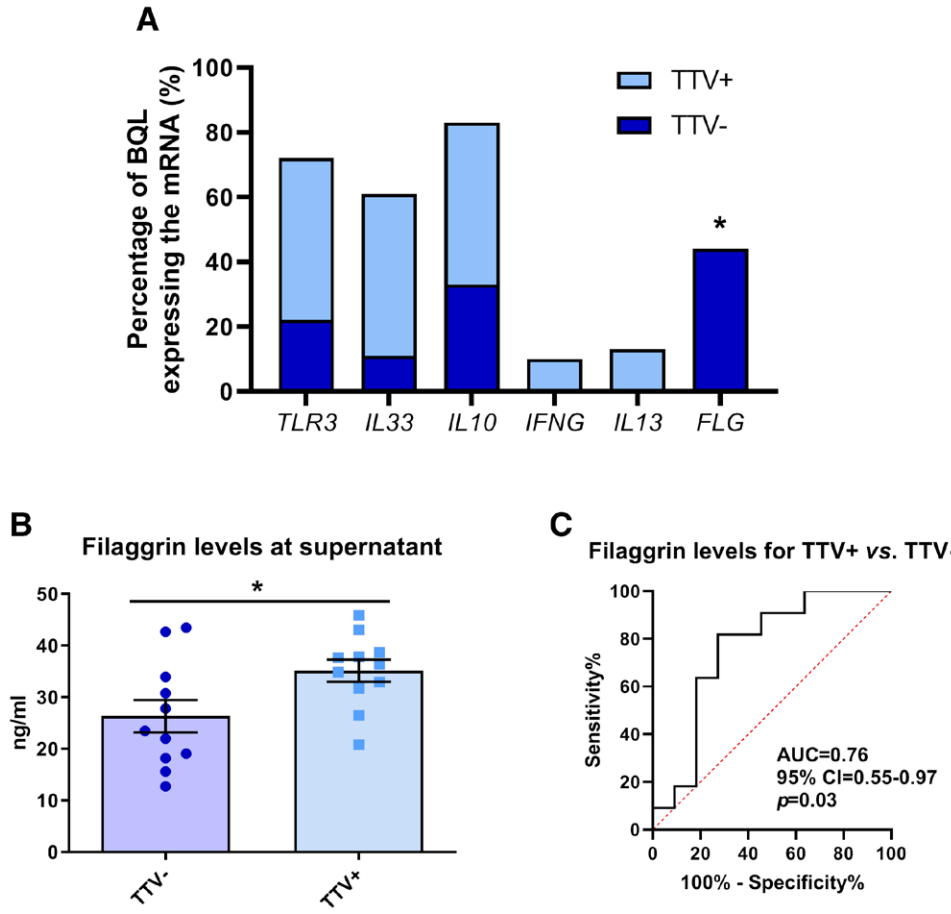

FIGURE 1. Filaggrin expression in infants with respiratory infections. Filaggrin expression is more frequent in infants without TTV, whereas its quantity in the supernatant is higher in the TTV+ group. (A) Frequency (%) of infants expressing the selected mRNA transcripts (TLR3, IL-33, IL-10, IFNG, IL-13 and FLG) detected by qPCR. (B) Quantity of filaggrin (ng/mL) in the supernatants of infants infected or not with TTV measured by ELISA. (C) ROC curve and AUC determination for filaggrin levels (ng/mL) in NPA supernatant as a TTV+ versus TTV– biomarker. *$P < 0.05$. ND indicates not detected; TTV, torque teno virus. full color online

pathophysiology of this association. The TTV+ patients tended to show higher expression of *TLR3* and *IL33* in the NPA, although the difference did not reach statistical significance, probably due to the small sample analyzed. However, these results suggest that there is a higher detection of genes upregulated by a viral infection in the group infected by TTV. The increased expression of *TLR3* and *IL-33* in NPA was previously described by our group, primarily in children with bronchiolitis, which was also the main diagnosis in our cohort.[25] Both, *TLR3* and *IL-33* have been found to be overexpressed in animal models of asthma exacerbation.[26] Some studies have shown that some viruses like RSV or HRV induce IL-33 synthesis by damaging epithelium in pulmonary diseases.[27]

The most important finding of our study is that fewer TTV+ children expressed filaggrin mRNA when compared with the TTV− ones, but in contrast, the filaggrin protein was increased in the NPA from these TTV+ children. Filaggrin is a protein involved in the integrity of the epithelial barrier and its mutations have been associated with atopic dermatitis or ichthyosis.[28,29] In our TTV+ patients, filaggrin is likely under-expressed, and its increase in NPA is part of the ongoing repair mechanism to maintain barrier function, triggered to respond to the epithelial damage caused by viral infection. Filaggrin release from keratohyalin granules into the keratinocyte cytoplasm is a main event in the cornification process, and it is critical for skin barrier function. The increase of filaggrin observed in the supernatant might be explained by damage to the cellular integrity and the consequent liberation of intracellular filaggrin to the extracellular medium.[30] Interestingly, filaggrin levels in supernatant might be used as TTV viral infection biomarkers.

Whether the presence of TTV in respiratory samples is a marker of a deficient immune response or is a consequence of it is difficult to elucidate. We cannot rule out that the previous presence of chronic TTV infection predisposes patients to infection by other CRVs and to greater severity.

On the other hand, the association of altered filaggrin with the possible development of asthma, as well as increased Th-2 immune response in our patients, who clinically had a diagnosis of bronchiolitis or recurrent wheezing, suggests that the presence of TTV infection could have a role in respiratory diseases. Several studies have suggested that TTV plays a role in the development and/or exacerbation of respiratory diseases in childhood such as asthma.[31] It has been postulated that TTV has a role in respiratory dysfunction, either alone or synergistically with other viruses, and could act as an enhancer of inflammation systemically or at specific body sites, such as the upper and lower airways.[32]

A limitation of our study is that it was performed during a pandemic caused by a respiratory virus. Thus, as has been previously mentioned, the sample size was small, and although plausible, it does not allow for firm conclusions to be drawn about the immune response. However, it provides a comprehensive view of TTV RI, which has been little studied so far, in a homogeneous population of infants.

In conclusion, TTV infection is common in children with viral RIs and could be associated with pneumonia and greater severity, as well as an alteration in the epithelial barrier due to low filaggrin gene expression. Larger prospective studies will be able to unravel whether TTV favors respiratory viral infections or is a marker of an impaired immune response.

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical assistance provided by Noelia Reyes; Vanessa Montero and Diana K. Santos in the Respiratory Viruses and Influenza Unit, CNM, ISCIII.

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