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Anellovirus loads are associated with outcomes in pediatric lung transplantation

Joshua A. Blatter, MD, MPH^{1, #}, Stuart C. Sweet, MD, PhD¹, Carol Conrad, MD², Lara A. Danziger-Isakov, MD, MPH³, Albert Faro, MD⁴, Samuel B. Goldfarb, MD⁵, Don Hayes Jr., MD, MS, MEd⁶, Ernestina Melicoff, MD⁷, Marc Schechter, MD⁸, Gregory Storch, MD¹, Gary A. Visner, DO⁹, Nikki M. Williams, BS¹⁰, and David Wang, PhD^{11, 12}

¹Department of Pediatrics, Washington University School of Medicine, St. Louis MO

²Department of Pediatrics, Stanford University School of Medicine, Stanford, CA

³Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

⁴Cystic Fibrosis Foundation, Bethesda, MD

⁵Department of Pediatrics, University of Pennsylvania, Philadelphia, PA

⁶Department of Pediatrics, The Ohio State University, Columbus, OH

⁷Department of Pediatrics, Baylor College of Medicine, Houston, TX

⁸Department of Pediatrics, University of Cincinnati, Cincinnati, OH

⁹Department of Pediatrics, Harvard Medical School, Boston, MA

¹⁰National Institutes of Health, Bethesda, MD

¹¹Department of Pathology and Immunology, Washington University School of Medicine, St. Louis MO

¹²Department of Molecular Microbiology, Washington University School of Medicine, Saint Louis MO

Abstract

Anelloviruses are DNA viruses ubiquitously present in human blood. Due to their elevated levels in immunosuppressed patients, anellovirus levels have been proposed as a marker of immune status. We hypothesized that low anellovirus levels, reflecting relative immunocompetence, would

#Correspondence and reprint requests to: Joshua A. Blatter, MD, MPH, Department of Pediatrics, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8116, Saint Louis, MO 63110, Phone: 314-454-2694, Fax: 314-454-2515, blatter@wustl.edu.

DR. JOSHUA A. BLATTER (Orcid ID : 0000-0002-4948-1023)

DR. LARA DANZIGER-ISAKOV (Orcid ID : 0000-0002-5691-5221)

DR. ERNESTINA MELICOFF (Orcid ID : 0000-0001-5696-7112)

Author contributions:

Study conception and design - JAB, SCS, DW

Recruitment of study subjects and acquisition of samples - JAB, SCS, CC, LAD, AF, SBG, DH, EM, MS, GS, GAV, NMW

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be associated with adverse outcomes in pediatric lung transplantation. We assayed blood samples from 57 patients in a multicenter study for alpha- and betatorquevirus, two anellovirus genera. The primary short-term outcome of interest was acute rejection, and longer-term outcomes were analyzed individually and as “composite” (death, chronic rejection, or retransplant within two years). Patients with low alphatorquevirus levels at two weeks post-transplantation were more likely to develop acute rejection within three months after transplant ($p=0.013$). Low betatorquevirus levels at six weeks and six months after transplant were associated with death ($p=0.047$) and the composite outcome ($p=0.017$), respectively. There was an association between low anellovirus levels and adverse outcomes in pediatric lung transplantation. Alphatorquevirus levels were associated with short-term outcomes (i.e., acute rejection) while betatorquevirus levels were associated with longer-term outcomes (i.e., death, or composite outcome within two years). These observations suggest that anelloviruses may serve as useful biomarkers of immune status and predictors of adverse outcomes.

INTRODUCTION

Significant advances in lung transplantation have occurred, including improvements in surgical technique, immunosuppressive regimens, and treatment of allograft rejection. Nevertheless, there have only been small increases in long-term survival outcomes in pediatric lung transplantation. The International Society for Heart and Lung Transplantation (ISHLT) registry reported no significant improvement in median survival in pediatric lung transplant recipients in 2006–2012 as compared to 2000–2005 (1). Both early and late transplant complications influence allograft survival. While chronic lung allograft dysfunction (CLAD) is the primary reason for limited advancements in survival, it is also understood that the early post-transplant course impacts long-term allograft outcomes. Acute cellular rejection (ACR) occurring in the early post-transplant period is associated with late allograft failure (2).

When compared to other transplanted solid organs, the lungs are distinguished by extensive antigenic exposure, and lung infection is associated with CLAD (3). Results of studies investigating an association between viral infection and episodes of acute rejection are mixed (4, 5). Nevertheless, regardless of whether viruses directly influence short- or long-term outcomes in transplant, they may also have indirect utility as a functional biomarker. A viral biomarker that is associated with levels of immune suppression, for example, could help influence treatment: patients who demonstrate minimal immune suppression could have dosing of their immunosuppressive medications optimized, and consideration could be given for preemptive surveillance or treatment of allograft rejection in these at-risk individuals (6).

The family *Anelloviridae*, a diverse group of small circular single-stranded DNA viruses, is inversely associated with immune status in a number of studies. Three anellovirus genera are known to infect humans: alphatorquevirus, betatorquevirus, and gammatorquevirus (7). Anelloviruses and anellovirus antibodies are widely prevalent in human populations at all ages (8, 9), leading to a presumption of commensality. Only limited information about anellovirus epidemiology is available. Further understanding of this virus would be dependent on improved culture methods (10) and serologic testing (11).

Nevertheless, while anellovirus is not known to cause human disease, high levels of anellovirus are associated with immune suppression, with low levels of anellovirus suggesting relative immunocompetence (12–17). Anelloviruses are inversely associated with levels of immunocompetence in HIV patients, with high plasma anellovirus loads being associated with low CD4 counts (13). Adult lung transplant recipients have increased levels of anellovirus in bronchoalveolar lavage fluid (14) and in serum (15). In addition, moderate-to-severe episodes of acute rejection in heart transplant recipients are associated with lower plasma anellovirus loads (15). Anelloviruses were recently identified as a potential marker of chronic rejection, with low plasma alphatorquevirus levels being associated with the subsequent development of CLAD (17).

While there is literature exploring anelloviruses in adult lung transplant recipients (14, 15), this study is the first to examine them in a pediatric cohort, and the first to incorporate both alpha- and betatorqueviruses. Given that anellovirus levels are inversely correlated with immunocompetence, we proposed that high levels of anellovirus in the blood would be associated with lower levels of rejection and improved longer-term outcomes. Moreover, it is reasonable to anticipate that different genera of anellovirus could help to predict different clinically relevant outcomes, given that the genera of anellovirus have varied self-protective mechanisms (18) and that anellovirus genera have differing patterns of early acquisition and subsequent prevalence (8). Genus-specific association with clinical outcomes was previously noted in a study of young febrile children, in whom alphatorqueviruses were found to correlate with fever while betatorqueviruses were not (10). We therefore sought to determine whether alpha- and betatorqueviruses could be associated with clinically relevant outcomes.

MATERIALS AND METHODS

Participant description

The participants in this study are part of the Clinical Trials in Organ Transplantation in Children (CTOT-C03) cohort (19). From June 2009 through January 2013, a total of 61 patients (age 0 to 18 years, average 11 years) were enrolled at six sites, including Lucile Packard Children's Hospital (Stanford), Boston Children's Hospital (Harvard), St. Louis Children's Hospital (Washington University), Nationwide Children's Hospital (Ohio State), Texas Children's Hospital (Baylor), Children's Hospital of Philadelphia (University of Pennsylvania). Among these 61 patients, 57 patients had sufficient collection of blood at two weeks, six weeks, and/or six months following transplant, to allow analysis for anellovirus load at one or more of the time points. Induction immunosuppression was site specific, while the protocol for maintenance immunosuppression (including tacrolimus, mycophenolate, and prednisone) was standardized by weight across all participating sites and standard-of-care antiviral prophylaxis was used. Surveillance lung biopsies were obtained routinely post-transplant at two weeks, one month, two months, three months, six months, nine months, one year, and 18 months, with additional biopsies obtained as clinically indicated. Clinical outcomes, including both short-term outcomes such as primary graft dysfunction and acute rejection, and longer-term outcomes such as CLAD, were tracked in these patients over a two-year period. This study was approved by the Washington University in St. Louis Institutional Review Board (ID201102281).

Blood preparation for Anellovirus PCR analysis

Nucleic acid was extracted from the whole blood samples using the bioMerieux EasyMag platform (bioMerieux, Boxtel, The Netherlands), using standard EasyMag reagents, including lysis buffer and quartz silica. A total of 200uL of whole blood was added to 2mL lysis buffer and vortexed. Quartz silica (100uL) was added to this mixture, which was again vortexed. The mixture was added to an EasyMag plastic strip well and placed on the extractor. Total elution volume was 100uL.

Anellovirus PCR analysis

TaqMan quantitative real-time PCR was performed to detect alpha- (12, 20) and betatorquevirus in extracted samples, using primers specific to each genus. The alphatorquevirus assay targeted a highly conserved segment of the viral untranslated region (20). Each alphatorquevirus reaction was performed in 25uL total volume, including 3uL extracted blood sample, TaqMan Fast Advanced Master Mix (Thermo Fisher), 0.9uM AMTS forward primer (5' GTGCCGIAGGTGAGTTTA 3'), 0.9uM AMTAS reverse primer (5' AGCCCGGCCAGTCC 3'), and 250nM AMTPTU probe (5' TCAAGGGGCAATTCGGGCT 3', 6-FAM/ZEN/3'IBFQ). Cycling conditions were 50C for 2 minutes and 95C for 20 seconds, followed by 40 cycles of 95C for 1 second and 60C for 20 seconds. The betatorquevirus assay targeted a short segment near the ORF 2 (21, 22). Each betatorquevirus reaction was performed in 20uL total volume, including 3uL extracted blood sample, TaqMan Fast Advanced Master Mix, 0.5uM forward primer (5' AGT TTA TGC CGC CAG ACG 3'), 0.5uM reverse primer (5' CCC TAG ACT TCG GTG GTT TC 3'), 250nM probe (5' ACT CAC CTH CGG CAC CCG C 3', 6-FAM/TAMRA). Cycling conditions were 95C for 20 seconds, followed by 40 cycles of 95C for 1 second and 60C for 20 seconds. All reactions were performed on a ViiA 7 Real-Time PCR System (Applied Biosystems) and were analyzed using default ViiA 7 Software v1.2 settings. Plasmid controls containing the target sequences for the alphatorquevirus and betatorquevirus assays were synthesized and used to generate a standard curve for each (alphatorquevirus curve: slope -3.67, R^2 0.99; betatorquevirus curve: slope -3.83, R^2 0.99). The detection limit for the alphatorquevirus assay was 30 copies, and for the betatorquevirus assay was 3 copies. For the purpose of analysis, samples below the limit of detection were assigned a value at the bottom of rank order—and for analyses in which a numeric value was needed, negative assays were assigned a copy number one-half the detection limit.

Definitions of clinical metadata

Race was self-reported, and “white” and “non-white” groups included both Hispanic and non-Hispanic respondents. For the purpose of the current analysis, patients were considered “non-white” if their race was identified as black, Asian, or unknown. All participating transplant centers shared a common protocol for the acquisition and interpretation of data related to transplant complications. Primary graft dysfunction (PGD) was identified by characteristic radiographic changes and decreased oxygenation (PaO₂/FiO₂ ratio), based on standard guidelines proposed by the ISHLT Working Group on Primary Lung Graft Dysfunction (23). Presence of PGD was assessed as Grade 2 or 3 disease at 48 hours or more following transplant. ACR was defined according to pathology specimens obtained

from transbronchial biopsy, based on the ISHLT consensus guidelines (24, 25), with presence assessed as Grade 1 disease or above. Bronchiolitis obliterans (BO) was identified by the presence of dense fibrous tissue in the small airways as found in transbronchial or open lung biopsies, based on standard guidelines (26). All pathology findings were reviewed by two pulmonary pathologists at active lung transplant centers, with discordant interpretations reviewed by a third, independent transplant pathologist. Bronchiolitis obliterans syndrome (BOS) diagnosis (26), identified by a characteristic decline (Grade 0-p or above) in pulmonary function testing (PFT), was applied only after graft infection and bronchial stricture were ruled out. PFT changes were reviewed by two blinded pediatric pulmonologists, with a third independent expert called upon to review discordant interpretations. All children identified as capable of performing PFTs had testing administered. The “composite endpoint,” intended to capture longer-term outcomes, was identified as present if a patient experienced death, BO/BOS, or retransplant within two years following transplant.

Statistical analyses

Alpha- and betatorquevirus levels at paired time points were compared with paired t-testing, and comparison among the three time points was assessed with a multivariate analysis of variance (MANOVA) model, adapted for use with repeated measures. Bivariate analyses of anellovirus levels and outcomes of interest were conducted with Fisher’s exact test, with a result of $p < 0.05$ considered statistically significant. Based on existing assessments of anellovirus levels in transplant patients, it was suspected that anellovirus levels would have a tendency to change over time (15) and that levels would have characteristic variability based on the interval following transplant. Therefore, in order to normalize anellovirus levels by time point (i.e., two weeks, six weeks, and six months following transplant)—comparable to the time-normalized anellovirus levels used in a prior study (15)—median alpha- and betatorquevirus levels were determined at each time point. A patient’s alpha- or betatorquevirus level could then be assessed as “high” or “low” depending on whether the patient was above or below a median level at the respective time point. A Kaplan-Meier curve was constructed to analyze the association between alphatorquevirus and ACR-free survival, with significance measured by log-rank testing. We also applied an adjusted logistic regression model to anellovirus and ACR in order to assess the effect of known or suspected covariates (such as patient age or transplant site). Potential association between alpha- and betatorquevirus levels was assessed by Spearman correlation.

If a patient achieved an outcome of interest (with the exception of PGD) prior to the measurement of an anellovirus level at a given time point, the time point was censored from that analysis. For example, in assessing the prognostic utility of a six-month betatorquevirus level for the composite outcome, only those patients who did meet the composite outcome between six months and 2 years post-transplant were included in the analysis. The number of patients and samples analyzed for each outcome at each time point, therefore, was limited by the availability of sample, and by whether the outcome of interest had already occurred prior to that time point.

RESULTS

Study participants

Key characteristics of the study participants who had sufficient collection of blood (from approximately two weeks to six months following transplant) to allow analysis for anellovirus load, are shown in Table 1. Patients were more likely to be white than non-white, and to be female than male. The most common indication for transplant was cystic fibrosis, affecting approximately half of the participants. More than one-third of patients experienced an episode of ACR within the first three months following transplant, and one-third of patients experienced the composite outcome (i.e., death, BO/BOS, retransplant) within two years following transplant. There was not a statistically significant correlation between paired alpha- and betatorquevirus levels (by Spearman correlation, $p>0.05$, analysis not shown). A complete set of data, including patient characteristics and anellovirus levels, is shown in Supplemental Table 1.

Anellovirus levels by time point

Figure 1 shows alpha- and betatorquevirus copy numbers by time point. Mean alphatorquevirus levels were higher at all time points (two weeks, six weeks, and six month copy numbers, respectively: 1.68×10^6 , 9.60×10^6 , 1.98×10^7) as compared to beta- levels (2.20×10^2 , 1.02×10^4 , 3.97×10^4). Both genera of anellovirus had a tendency toward increase over time. The repeated measures analysis of alphatorquevirus levels showed significant difference between time points ($p=0.030$), while the comparison among time points for betatorquevirus was not significant.

Anellovirus levels at two weeks following transplant

The results of bivariate analyses of alphatorquevirus levels at approximately two weeks (average 11 days) following transplant are shown in Table 2. There was a significant association between race and alphatorquevirus levels ($p=0.002$), and between age and alphatorquevirus levels ($p<0.001$). All non-white transplant recipients (9/9, 100%) had above median alphatorquevirus levels. The most common indications for transplant were cystic fibrosis, pulmonary vascular disease, and bronchiolitis obliterans, and there was not an association between diagnosis and anellovirus levels. There was a significant association between ACR and alphatorquevirus levels, with patients with below-median alphatorquevirus levels at two weeks following transplant being more likely to develop an episode of ACR within the first three months following transplant ($p=0.013$). There was no association between alphatorquevirus and any of the longer-term outcomes or with episodes of ACR that occurred more than 3 months post-transplant. The comparable analysis for betatorquevirus did not show any association between immediate post-transplant levels and short- or longer-term outcomes (analysis not shown). A Kaplan-Meier survival curve (representing “ACR-free survival”) ($p=0.0042$) also demonstrated a clear association with alphatorquevirus load in Figure 2. Given that there was a bivariate association between alphatorquevirus load and both race and age, a multivariable logistic regression model incorporating these covariates is displayed in Supplemental Table 2: after adjustment, individuals with a low alphatorquevirus load (at two weeks following transplant) had more than seventeen times the odds of having an episode of ACR within three months of

transplant ($p=0.021$). In order to account for potential site- or disease-specific differences in care, including induction schemes, this model was additionally adjusted for transplant site and pre-transplant diagnosis (neither of which was found to be significant in the multivariable model). There was a significant difference ($p=0.014$) between log-adjusted alphatorquevirus levels at two weeks following transplant, comparing patients who did and did not develop early ACR (Supplemental Figure 1). In an exploratory analysis, tacrolimus levels at two weeks following transplant were not associated with the development of ACR ($p>0.05$) and there was not a significant association between tacrolimus levels and alphatorquevirus copy number (Spearman correlation -0.16 , $p>0.05$) (analysis not shown).

Anellovirus levels at six weeks following transplant

Table 3 shows bivariate analyses of betatorquevirus levels at approximately six weeks (average 44 days) following transplant. There was a significant association between age and betatorquevirus levels at this time point ($p=0.015$). There was no association between betatorquevirus levels and episodes of ACR within the first three months following transplant, but there was an association ($p=0.047$) between betatorquevirus levels and death within two years following transplant. All patients who died within two years of transplant (5/5, 100%) had below-median betatorquevirus levels at six weeks following transplant. There was a significant difference ($p=0.022$) between log-adjusted betatorquevirus levels at six weeks following transplant, comparing patients who did and did not die within two years of transplant (analysis not shown). The comparable alphatorquevirus analysis did not show any association between six-week post-transplant levels and short- or longer-term outcomes (analysis not shown).

Anellovirus levels at six months following transplant

Bivariate analyses of betatorquevirus levels at approximately six months (average 181 days) following transplant are shown in Table 4. All longer-term outcomes (death, chronic rejection, retransplant) showed analogous trends, with lower levels of betatorquevirus being associated with adverse outcomes. The composite outcome, which incorporates all three of these outcomes, was significantly associated with below-median betatorquevirus levels at six months post-transplant ($p=0.017$). There was a significant difference ($p=0.047$) between log-adjusted betatorquevirus levels at six months following transplant, comparing patients who did and did not develop the composite outcome within two years of transplant. The association between betatorquevirus and the composite outcome was not impacted by the addition of patient age in a multivariable model (analysis not shown). In a comparable alphatorquevirus analysis, there was no association with the composite outcome ($p=0.31$) or with any individual longer-term outcome (analyses not shown).

DISCUSSION

In this study, we determined that blood anellovirus levels were associated with key clinical outcomes in pediatric lung transplant recipients. Alphatorquevirus levels were associated with episodes of ACR while betatorquevirus levels were associated with longer-term outcomes including death, retransplant, and BO/BOS. This study is the first to report an association between anellovirus levels and both short- and longer-term outcomes in pediatric

lung transplantation. Of particular note is the finding that distinct lung transplant outcome associations were defined with different anellovirus genera. Low anellovirus levels—whether alphatorquevirus levels in the immediate post-transplant period, or betatorquevirus in the months following transplant—may reflect a state of relative immunocompetence that predisposes patients to rejection or allograft failure.

Anellovirus levels have been associated with transplant immunosuppression in multiple previous reports. In a study of 80 liver transplant recipients and 19 healthy children, serum anellovirus levels increased substantially after transplant as compared to prior to transplant ($p<0.001$), and as compared to healthy controls ($p<0.0001$) (16). Average alphatorquevirus loads in that study (approximately 10^5 copies per mL) were somewhat lower than those observed in the current cohort (approximately 10^6 – 10^7).

There have been some associations noted between anellovirus levels and clinical outcomes of interest in solid organ transplant. Low blood anellovirus loads were reported in heart transplant recipients with moderate to severe graft rejection, as compared to non-rejecting patients ($p=0.011$) (15). Patients who developed primary graft dysfunction (PGD) after lung transplantation underwent a smaller increase in anellovirus levels from before to after transplant, as compared to non-PGD controls ($p=0.0026$) (27). In another study of lung transplant recipients, alphatorquevirus levels assessed prior to the diagnosis of CLAD were significantly lower in patients who subsequently developed CLAD ($p=0.020$) (17). While no association between alphatorqueviruses and CLAD (defined in the current analysis as either BO or BOS) was established in the current study, the different results may be due to differences in the cohorts or in the timing of the samples tested.

There have only been a few prior efforts to identify associations between clinical outcomes and discrete anellovirus taxa. Distinct phylogenetic clusters of alphatorquevirus were found in patients prior to liver transplant and healthy controls, as compared to patients following liver transplant ($p=0.039$) (16). In a study of children between the ages of 2–36 months, alphatorquevirus was more prevalent in nasopharyngeal swab specimens of febrile children as compared to healthy controls ($p=0.018$) while betatorquevirus was equally prevalent in both groups (10).

Overall, it is not yet clear whether anelloviruses are primarily bystanders in disease states, or whether they have clinically relevant immunomodulatory qualities or direct pathogenic potential. Anelloviruses have been demonstrated to impact a host's antiviral immunity *in vitro*. For example, alphatorqueviruses encode miRNAs that can inhibit the interferon response (18). However, miRNA candidates have not been found among beta- or gammatorqueviruses, which could reflect the relative dearth of information about these genera or could also suggest that they have in fact developed different protective mechanisms (18). Furthermore, some anelloviruses are capable of interfering with NF-kappaB signaling via both canonical and non-canonical pathways, thereby inhibiting interleukin-6 (IL-6) and IL-8 transcription (28). While anelloviruses can inhibit immune function, they also stimulate innate immune response. In an *in vitro* study, alphatorqueviruses stimulated murine spleen cells to produce interferon-gamma and IL-10, via a toll-like receptor (TLR)-9 mediated mechanism (29). Anellovirus has not only been

implicated in triggering innate immune function, but adaptive immunity as well. Early anellovirus studies involving serum screening showed that anellovirus antibodies were present in nearly all participants, suggesting a sustained human immune response to anellovirus infection (9).

The tissue tropism of anellovirus is poorly understood, but hematopoietic stem cells have been tentatively identified as the primary source of high anellovirus burden, based on data from four leukemia patients who underwent stem cell transplant (30). While immunosuppressive states contribute to anellovirus growth, expansion of anellovirus is not immediate in the post-transplant period. In a study of 46 lung transplant recipients, the median peak alphatorquevirus level occurred at a median time of 67 days (range 41–92 days) (31). A comparable lag in the development of increased anellovirus levels following solid organ transplant has been described (15), and occurred in the current study as anellovirus levels increased between two weeks and six months post-transplant (Figure 1). The gradual expansion of anellovirus could help to account for the variable utility of genera-specific counts at different intervals following transplant: immediate post-transplant anellovirus levels could reflect donor characteristics (27) and may not yet fully reflect recipient host immunity, given that steady state populations of anellovirus are not achieved for approximately two months (31). Alternately, immediate post-transplant anellovirus levels could reflect recipient characteristics: alphatorquevirus doubling times in the first two months after transplant reflect pre-transplant levels ($p < 0.001$) (31). If immediate post-transplant alphatorquevirus levels both reflect pre-transplant levels and predict ACR, it is reasonable to consider whether patients who are likely to experience ACR can be identified even *before* transplant.

Nevertheless, transplant recipient pharmacogenomics could account in part for the high alphatorquevirus levels observed at the two week time point among non-white respondents in the current study: genetic polymorphisms (e.g., CYP3A4) that have a characteristic race-specific distribution and which may impact tacrolimus metabolism, were previously described (32). Moreover, in a study of the Cylex Immune Cell Function assay (a functional test of immune status occasionally used in lung transplant recipients), African-Americans had lower immune function measures at all post-transplant time points as compared to non-African-Americans (33). The significant association between age and anellovirus levels in the current study (described previously (8)) did not have an impact on the association between anellovirus and clinical outcomes of interest (e.g., Supplemental Table 2), but offers an intriguing view of anellovirus population dynamics that will need to be explored further in future studies.

The association of anellovirus genera with varied clinical outcomes, both in prior studies (10, 16) and in the present one, suggests that the anellovirus family has multiple mechanisms for impacting immunity, or that the host immune control of anelloviruses varies by genus. Betatorquevirus may not be useful for clinical prediction early after transplant because it is relatively scarce (as compared to alphatorquevirus), or because betatorquevirus levels do not reflect the immune processes relevant to short-term outcomes: given that the mechanism of acute cellular rejection has been assumed to be T cell recognition of human leukocyte

antigen (HLA) complexes (34), for example, it is possible that alpha-, more accurately than betatorqueviruses, reflect T cell kinetics in the early post-transplant period.

An important limitation of the current study is the small sample size. However, it represents a substantial percentage of pediatric lung transplants performed in the United States over this time period, given that only 34 children underwent lung transplant in 2012 (35). In addition, the current study does not directly explore whether anellovirus has a causative role in the development of disease. With the study design being retrospective, the precise temporal relationship between anellovirus expansion and adverse outcomes could not be determined. Although multicenter trials can display a site-specific bias, transplant site did not impact the association between anellovirus and ACR (Supplemental Table 2). It is worth emphasizing that the current study employed genus-level consensus primers that were not intended to identify species-level differences between samples. Prospective studies with higher resolution sampling would allow us to draw further conclusions about the dynamics of anellovirus populations, and to find potential associations with other key transplant outcomes.

The investigation of anellovirus in transplantation is very limited with only one study explicitly examining anellovirus as a biomarker of immunosuppression (31). Nevertheless, based on the findings of the current study, we propose that anellovirus has utility not only for estimating immune suppression, but also for predicting clinical outcomes. Future study should include an in-depth analysis of associations between anellovirus and other viruses or bacteria, and between anellovirus and other clinical outcomes. Given the apparent utility of early post-transplant anellovirus levels, pre-transplant levels should be measured in a future study as it is possible they could be used to stratify recipient risk. Transplant recipients deemed to be “at risk” of adverse outcomes due to low anellovirus levels could have immune suppression increased, but could also undergo prophylactic treatment (e.g., starting azithromycin for CLAD in a patient with low betatorquevirus levels).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACR	acute cellular rejection
BO	bronchiolitis obliterans
BOS	bronchiolitis obliterans syndrome
CLAD	chronic lung allograft dysfunction

PCR	polymerase chain reaction
PGD	primary graft dysfunction

References

1. Benden C, Goldfarb SB, Edwards LB, et al. The Registry of the International Society for Heart and Lung Transplantation: Seventeenth Official Pediatric Lung and Heart–Lung Transplantation Report —2014; Focus Theme: Retransplantation. *The Journal of Heart and Lung Transplantation*. 2014; 33:1025–1033. [PubMed: 25242126]
2. Bando K, Paradis IL, Similo S, et al. Obliterative bronchiolitis after lung and heart-lung transplantation. An analysis of risk factors and management. *J Thorac Cardiovasc Surg*. 1995; 110:4–13. discussion 13–14. [PubMed: 7609567]
3. Heng D, Sharples LD, McNeil K, Stewart S, Wreghitt T, Wallwork J. Bronchiolitis obliterans syndrome: incidence, natural history, prognosis, and risk factors. *J Heart Lung Transplant*. 1998; 17:1255–1263. [PubMed: 9883768]
4. Vu DL, Bridevaux PO, Aubert JD, Soccal PM, Kaiser L. Respiratory viruses in lung transplant recipients: a critical review and pooled analysis of clinical studies. *Am J Transplant*. 2011; 11:1071–1078. [PubMed: 21521473]
5. Peghin M, Hirsch HH, Len O, et al. Epidemiology and Immediate Indirect Effects of Respiratory Viruses in Lung Transplant Recipients: A 5-Year Prospective Study. *Am J Transplant*. 2017; 17:1304–1312. [PubMed: 27615811]
6. Fernandez-Ruiz M, Kumar D, Humar A. Clinical immune-monitoring strategies for predicting infection risk in solid organ transplantation. *Clin Transl Immunology*. 2014; 3:e12. [PubMed: 25505960]
7. Li L, Deng X, Da Costa AC, Bruhn R, Deeks SG, Delwart E. Virome analysis of antiretroviral-treated HIV patients shows no correlation between T-cell activation and anelloviruses levels. *J Clin Virol*. 2015; 72:106–113. [PubMed: 26479202]
8. Ninomiya M, Takahashi M, Nishizawa T, Shimosegawa T, Okamoto H. Development of PCR Assays with Nested Primers Specific for Differential Detection of Three Human Anelloviruses and Early Acquisition of Dual or Triple Infection during Infancy. *Journal of Clinical Microbiology*. 2008; 46:507–514. [PubMed: 18094127]
9. Ott C, Duret L, Chemin I, Trepo C, Mandrand B, Komurian-Pradel F. Use of a TT virus ORF1 recombinant protein to detect anti-TT virus antibodies in human sera. *J Gen Virol*. 2000; 81:2949–2958. [PubMed: 11086126]
10. McElvania TeKippe E, Wylie KM, Deych E, Sodergren E, Weinstock G, Storch GA. Increased Prevalence of Anellovirus in Pediatric Patients with Fever. *PLoS ONE*. 2012; 7:e50937. [PubMed: 23226428]
11. Mankotia DS, Irshad M. Cloning and expression of N22 region of Torque Teno virus (TTV) genome and use of peptide in developing immunoassay for TTV antibodies. *Virol J*. 2014; 11:96. [PubMed: 24884576]
12. Walton AH, Muenzer JT, Rasche D, et al. Reactivation of multiple viruses in patients with sepsis. *PLoS One*. 2014; 9:e98819. [PubMed: 24919177]
13. Shibayama T, Masuda G, Ajisawa A, et al. Inverse relationship between the titre of TT virus DNA and the CD4 cell count in patients infected with HIV. *AIDS*. 2001; 15:563–570. [PubMed: 11316992]
14. Young JC, Chehoud C, Bittinger K, et al. Viral metagenomics reveal blooms of anelloviruses in the respiratory tract of lung transplant recipients. *Am J Transplant*. 2015; 15:200–209. [PubMed: 25403800]
15. De Vlaminc I, Khush KK, Strehl C, et al. Temporal response of the human virome to immunosuppression and antiviral therapy. *Cell*. 2013; 155:1178–1187. [PubMed: 24267896]
16. Beland K, Dore-Nguyen M, Gagne MJ, et al. Torque Teno virus in children who underwent orthotopic liver transplantation: new insights about a common pathogen. *J Infect Dis*. 2014; 209:247–254. [PubMed: 23922368]

17. Görzer I, Jaksch P, Strassl R, Klepetko W, Puchhammer-Stöckl E. Association between plasma torque teno virus level and chronic lung allograft dysfunction after lung transplantation. 2016
18. Kincaid RP, Burke JM, Cox JC, de Villiers EM, Sullivan CS. A human torque teno virus encodes a microRNA that inhibits interferon signaling. *PLoS Pathog.* 2013; 9:e1003818. [PubMed: 24367263]
19. Sweet SC. Multicenter collaboration in pediatric lung transplantation--the importance of consensus. *Pediatr Transplant.* 2013; 17:197–198. [PubMed: 23448310]
20. Maggi F, Pifferi M, Fornai C, et al. TT virus in the nasal secretions of children with acute respiratory diseases: relations to viremia and disease severity. *J Virol.* 2003; 77:2418–2425. [PubMed: 12551979]
21. Moen EM, Sleboda J, Grinde B. Real-time PCR methods for independent quantitation of TTV and TLMV. *J Virol Methods.* 2002; 104:59–67. [PubMed: 12020793]
22. Garcia-Alvarez M, Berenguer J, Alvarez E, et al. Association of torque teno virus (TTV) and torque teno mini virus (TTMV) with liver disease among patients coinfecting with human immunodeficiency virus and hepatitis C virus. *Eur J Clin Microbiol Infect Dis.* 2013; 32:289–297. [PubMed: 22983402]
23. Christie JD, Carby M, Bag R, Corris P, Hertz M, Weill D. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part II: definition. A consensus statement of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant.* 2005; 24:1454–1459. [PubMed: 16210116]
24. Snell GI, Boehler A, Glanville AR, et al. Eleven years on: a clinical update of key areas of the 1996 lung allograft rejection working formulation. *J Heart Lung Transplant.* 2007; 26:423–430. [PubMed: 17449409]
25. Yousem SA, Berry GJ, Cagle PT, et al. Revision of the 1990 working formulation for the classification of pulmonary allograft rejection: Lung Rejection Study Group. *J Heart Lung Transplant.* 1996; 15:1–15. [PubMed: 8820078]
26. Estenne M, Maurer JR, Boehler A, et al. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. *J Heart Lung Transplant.* 2002; 21:297–310. [PubMed: 11897517]
27. Abbas AA, Diamond JM, Chehoud C, et al. The Perioperative Lung Transplant Virome: Torque Teno Viruses are Elevated in Donor Lungs and Show Divergent Dynamics In Primary Graft Dysfunction. *American Journal of Transplantation.* 2016 n/a–n/a.
28. Zheng H, Ye L, Fang X, et al. Torque teno virus (SANBAN isolate) ORF2 protein suppresses NF-kappaB pathways via interaction with IkappaB kinases. *J Virol.* 2007; 81:11917–11924. [PubMed: 17686849]
29. Rocchi J, Ricci V, Albani M, et al. Torquetenovirus DNA drives proinflammatory cytokines production and secretion by immune cells via toll-like receptor 9. *Virology.* 2009; 394:235–242. [PubMed: 19765789]
30. Maggi F, Focosi D, Albani M, et al. Role of hematopoietic cells in the maintenance of chronic human torquetenovirus plasma viremia. *J Virol.* 2010; 84:6891–6893. [PubMed: 20410268]
31. Gorzer I, Jaksch P, Kundi M, Seitz T, Klepetko W, Puchhammer-Stöckl E. Pre-transplant plasma Torque Teno virus load and increase dynamics after lung transplantation. *PLoS One.* 2015; 10:e0122975. [PubMed: 25894323]
32. Provenzano A, Santeusano A, Mathis E, et al. Pharmacogenetic considerations for optimizing tacrolimus dosing in liver and kidney transplant patients. *World J Gastroenterol.* 2013; 19:9156–9173. [PubMed: 24409044]
33. Bhorade SM, Janata K, Vigneswaran WT, Alex CG, Garrity ER. Cylex ImmuKnow assay levels are lower in lung transplant recipients with infection. *J Heart Lung Transplant.* 2008; 27:990–994. [PubMed: 18765191]
34. Martinu T, Chen DF, Palmer SM. Acute rejection and humoral sensitization in lung transplant recipients. *Proc Am Thorac Soc.* 2009; 6:54–65. [PubMed: 19131531]
35. Valapour M, Skeans MA, Heubner BM, et al. OPTN/SRTR 2012 Annual Data Report: Lung. *American Journal of Transplantation.* 2014; 14:139–165. [PubMed: 24373171]

Figure 1.

Alpha- and betatorquevirus copy number comparisons between paired time points. Alphatorquevirus levels at week 2 and month 6 (n=38) were significantly different as shown, while no paired comparisons between betatorquevirus time points (n=38) were significantly different. Average copy numbers of alphatorquevirus were higher than those of beta-. There was a general trend toward increase over these time points, although a repeated measures comparison was statistically significant for alphatorquevirus only (p=0.030), not for betatorquevirus. Error bars represent standard deviation from the mean.

Figure 2.

Kaplan-Meier survival curve (representing “ACR-free survival”), following all participants over the first three months following transplant. The assessment of “below” or “above” median alphatorquevirus levels was made on the basis of copy number at approximately 2 weeks following transplant (n=48).

Table 1

Participant characteristics among patients with blood collection from approximately two weeks to six months following transplant to allow for anellovirus analysis (total N=57). Composite outcome includes death, BO/BOS, and retransplant.

	N (%)
Female sex	34 (60%)
White race	48 (84%)
<i>Age</i>	
<5 years	8 (14%)
5 to 12 years	15 (26%)
>12 years	34 (60%)
<i>Indication for transplant</i>	
Cystic fibrosis	29 (51%)
Primary pulmonary hypertension and other pulmonary vascular disease	10 (18%)
Bronchiolitis obliterans	10 (18%)
Other diagnosis	8 (14%)
Primary graft dysfunction	21 (37%)
Acute cellular rejection within three months	20 (36%)
Bronchiolitis obliterans within two years	6 (10%)
Bronchiolitis obliterans syndrome within two years	6 (10%)
Retransplant within two years	2 (4%)
Death within two years	9 (16%)
Composite outcome within two years	19 (33%)

Table 2

Characteristics of study participants, by alphatorquevirus status (with “high” representing above-median count numbers, and “low” representing below-median count numbers) at approximately two weeks following transplant (* representing $p < 0.05$ for bivariate comparison) (total N=47).

	High Alpha- (N=24)	Low Alpha- (N=23)
Female sex	16	15
White race*	15	23
<i>Age*</i>		
<5 years	6	0
5 to 12 years	10	3
>12 years	8	20
<i>Indication for transplant</i>		
Cystic fibrosis	8	16
Primary pulmonary hypertension and other pulmonary vascular disease	5	1
Bronchiolitis obliterans	6	4
Other diagnosis	5	2
<i>Induction therapy</i>		
Anti-IL-2	17	20
rATG	6	3
None	1	1
Primary graft dysfunction	10	6
Acute cellular rejection within three months*	4	13
Bronchiolitis obliterans within two years	3	3
Bronchiolitis obliterans syndrome within two years	3	2
Retransplant within two years	2	0
Death within two years	6	3
Composite outcome within two years	10	8

Table 3

Characteristics of study participants, by betatorquevirus status (with “high” representing above-median count numbers, and “low” representing below-median count numbers) at approximately six weeks following transplant (* representing $p < 0.05$ for bivariate comparison) (total N=40).

	High Beta- (N=20)	Low Beta- (N=20)
Female sex	13	12
White race	17	15
<i>Age*</i>		
<5 years	3	3
5 to 12 years	10	2
>12 years	7	15
<i>Indication for transplant</i>		
Cystic fibrosis	10	11
Primary pulmonary hypertension and other pulmonary vascular disease	3	3
Bronchiolitis obliterans	2	5
Other diagnosis	5	1
<i>Induction therapy</i>		
Anti-IL-2	15	14
rATG	3	5
None	2	1
Primary graft dysfunction	7	3
Bronchiolitis obliterans within two years	2	4
Bronchiolitis obliterans syndrome within two years	3	2
Retransplant within two years	2	0
Death within two years*	0	5
Composite outcome within two years	5	9

Table 4

Characteristics of study participants, by betatorquevirus status (with “high” representing above-median count numbers, and “low” representing below-median count numbers) at approximately six months following transplant (* representing $p < 0.05$ for bivariate comparison) (total N=46).

	High Beta- (N=23)	Low Beta- (N=23)
Female sex	11	18
White race	18	21
<i>Age</i>		
<5 years	5	1
5 to 12 years	9	5
>12 years	9	17
<i>Indication for transplant</i>		
Cystic fibrosis	12	12
Primary pulmonary hypertension and other pulmonary vascular disease	3	5
Bronchiolitis obliterans	2	4
Other diagnosis	6	2
<i>Induction therapy</i>		
Anti-IL-2	18	19
rATG	3	3
None	2	1
Bronchiolitis obliterans within two years	1	4
Bronchiolitis obliterans syndrome within two years	1	4
Retransplant within two years	0	1
Death within two years	0	3
Composite outcome within two years*	2	10