Journal of Clinical Virology 61 (2014) 329–333

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

# Prospective study on Norovirus infection among allogeneic stem cell transplant recipients: Prolonged viral excretion and viral RNA in the blood

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ARTICLE INFO

*Article history:*
Received 10 February 2014
Received in revised form 17 July 2014
Accepted 5 August 2014

*Keywords:*
Norovirus infection
Norovirus excretion
Calicivirus
Stem cell transplant
Viremia

ABSTRACT

*Background:* Human caliciviruses (*Norovirus* and *Sapovirus*) are important acute gastroenteritis agents. The Norovirus (NoV) disease is usually self-limited; however, prolonged viral excretion and complications have been reported, mainly in immunosuppressed individuals.
*Objectives:* In this prospective study, we have monitored allogeneic stem cell transplant (ASCT) patients for human calicivirus infection.
*Study design:* Ten ASCT patients were monitored for NoV and sapoviruses (SaV) infection, for a period of five months to a maximum of one year. Prolonged NoV excretion and long term viral RNA in the blood were assessed by multiplex RT-PCR targeting region C of the viral capsid. Secretor status of the patients was determined by enzyme immunoassay using Ulex Europaeus agglutinin. Partial genomic sequencing and phylogenetic analysis were performed to characterize the viral genotypes.
*Results:* NoV was detected in six out of ten patients (60%). Prolonged viral excretion in feces (mean of 61.6 days) and long term presence of NoV RNA in the sera (mean of 33.6 days) of the patients were observed. SaV was not detected in any of the samples. All patients had diarrhea, vomiting and fever during NoV positivity. All NoV-positive samples were characterized as GI.3 NoV. Three Nov-infected patients presented with acute intestinal graft versus host disease.
*Conclusions:* This study brings important information on NoV course of infection in ASCT patients. It also provides evidence for long term viral RNA in the blood highlighting the importance of the inclusion of NoV screening in the routine testing performed before transplantation and during follow-up of these patients.



## 1. Background

The *Norovirus* (NoV) and *Sapovirus* (SaV) are the only genera of the *Caliciviridae* family that have been associated with infection in humans [1,2]. These agents are transmitted by the fecal-oral route, through person-to-person contact, by ingestion of contaminated food or water [3,4], via airborne viral particles from vomit of infected patients, and by fomites [5]. Although some infected individuals may excrete a high number of viral particles in their feces, the infectious dose is low [6].

The NoV infection is usually self-limited. Its main symptoms are vomit, diarrhea and nausea [7,8]. Infection in hospitalized and/or immunocompromised patients can result in prolonged viral excretion and complications [9,10]. Data on NoV prolonged excretion by cancer patients is usually determined through retrospective analysis of outbreak samples. NoV viremia in these patients has been, generally, poorly assessed as well. Currently human calicivirus (HuCV) screening is not included in routine laboratory exams for cancer patients. Some prior studies have also reported that the symptoms of NoV infection may mimic those of the graft versus host disease (GVHD). This complication is yet another common occurrence that can lead to underreported cases of NoV among

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http://dx.doi.org/10.1016/j.jcv.2014.08.004
1386-6532/

allogeneic stem cell transplant (ASCT) patients [11,12]. This can result in an increase of drug induced immunosuppression leading to lethal complications [13].

This article discusses a recent prospective study aimed at the monitoring of HuCV occurrence among a group of ASCT recipients.

## 2. Objectives

Screening for HuCV occurrence among ASCT recipients, identifying any case of prolonged viral excretion, determining the long-term presence of viral RNA in the blood of NoV-positive patients, and identifying the genotype of any detected NoV or SaV viral strains.

## 3. Study design

### 3.1. Description of the Bone Marrow Transplant Unit (BMTU)

The BMTU of Hospital Araújo Jorge/Association for Combating Cancer in Goiás (HAJ/ACCGO) is the only referral unit, accredited by the Brazilian Ministry of Health, to perform allogeneic and autologous stem cell transplants through the Unified Health System (SUS) in the Central West region of Brazil. For the last three years an annual mean of 12 ASCT procedures have been conducted in the unit.

The BMTU is a 4-bed unit four private rooms numbered 217–220, in which each room is connected by a hallway that is preceded by the BMTU nursing station. One door separates each room from the hallway. All rooms are equipped with HEPA filters and have individual bathrooms and sinks.

All meals are provided by the hospital kitchen. Patients consume only bottled mineral water that is provided by the hospital.

### 3.2. Characteristics of the study population

This prospective study aims at the monitoring of patients in the BMTU of the HAJ/ACCGO for HuCV infection (NoV and SaV) for a minimum of five months to a maximum of a one-year period. From October 2012 to September 2013, a total of 12 ASCT procedures were performed in the BMTU. From that group, ten patients agreed to participate, and each was enrolled in this study after signing its consent form. Questionnaires regarding patients' symptoms were also filled out daily by the staff of the BMTU and reviewed weekly by the assigned physician. Ethical approval was obtained from the Research Ethics Committees of the ACCGO: 108.396; and of the Federal University of Goiás: 429/2011.

Forty-percent of the patients were male and 60 percent female. Patients' ages varied from 21 to 55 years. All of them received prophylaxis for GVHD with cyclosporine and methotrexate; antimicrobial prophylaxis with trimethoprim-sulfamethoxazole; fluconazole; acyclovir; and albendazole.

### 3.3. Data and sample collection

One week before their individual ASCT procedure, one sample each of feces and blood (serum) was obtained from each participant of the study, except for one patient (patient 3) from whom samples could be obtained only after her transplant. Saliva samples were also collected for determination of the secretor status.

After the procedure, one fecal sample per week was obtained from each patient, except in any event of patient's constipation. In any case where the patient presented with diarrhea, samples were obtained daily or every other day, while symptoms lasted. Blood samples were obtained every two weeks from each patient during the period of the study.

After discharged from the hospital, patients returned at variable intervals for outpatient clinic consultations. Samples were then obtained during those routine consultations.

### 3.4. Methodology

#### 3.4.1. Sample processing

All samples were kept at 4 °C until processed (always within two hours maximum). Saliva samples were collected from each patient in a sterile collector. Sera samples were separated by centrifugation at 2500 × *g*, after the clot retraction of the blood samples. Stool suspensions (20%) were prepared in PBS. After processing, samples were stored at −20 °C and −70 °C until they were tested.

### 3.5. Determination of the secretor status and Lewis phenotype

Histo-blood groups of the patients were obtained from patients' hospital records. The secretor phenotype was determined by an enzyme immunoassay, using Lectina-UEA (Ulex europaeus agglutinin) specific for Fucα1-2Gal-R present in secretor (but not non-secretor) saliva, according to Nordgren et al. [14]. Two known secretor-positive and two known secretor-negative saliva samples were always run in each plate as a control. All samples were run in triplicate.

#### 3.5.1. Nucleic acid extraction

Nucleic acids were extracted from stool suspensions using the method described by guanidine isothiocyanate/silica method [15,16]. For sera samples a commercial kit was used (QIAamp Viral RNA Mini kit-Qiagen, Freigburg, Germany). After extraction, cDNA synthesis was performed using a pd[N]6 TM random primer (Amersham Bioscience, Hilden, Germany).

#### 3.5.2. Human Calicivirus detection and genotyping

Screening for NoV and SaV was done by reverse transcription-multiplex polymerase chain reaction (RT-multiplexPCR). For genotyping the NoV and SaV a partial region of the capsid gene was amplified and sequenced. In RT-multiplexPCR, three pairs of specific primers: GISKF and GISKR for NoV genogroup I (GI NoV); G2SKF and G2SKR for NoV genogroup II (GII NoV); SLV5317 and SLV5749 for SaV were mixed [17,18]. Samples that were positive in the multiplex PCR were confirmed in a monoplex PCR, using all three pairs of primers in separate. The RT-multiplex PCR and monoplex PCR were performed according to the methods described by Kojima et al. and Yan et al., with modifications [19]. In all reactions positive (NoV and SaV previously sequenced samples) and negative (MilliQ water) were used in all steps of the process.

Samples from each patient were manipulated separately, and feces and sera were also tested separately. In order to decrease RT-PCR inhibition, all negative samples in a first RT-PCR round were processed again; diluted 1:2 in PBS; and viral RNA extraction and multiplex RT-PCR was repeated. To avoid cross-contamination, all procedures were performed in different PCR cabinets, located in separate rooms, with UV light decontamination before and after each step.

#### 3.5.3. Norovirus genomic sequencing and phylogenetic analysis

All 330 bp amplicons were purified using the QIAquick PCR Purification Kit (Qiagen Freigburg, Germany), and sequenced with the same primer pairs used for PCR amplification. Sequencing reaction were performed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequences quality analysis was determined by the Phred program [20]. Consensus sequences, obtained by alignment, were compared with other sequences deposited in the GeneBank, using the Clustal X software [21]. The