# Molecular Genotyping of Rotavirus Associated with Type 1 Diabetes Mellitus in Iraqi Children

Noor A. Oohayeed[1], Hassan M. Naïf[2]*

[1,2]*Department of Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrain University, Aljadriyah, Baghdad, Iraq.*

## ABSTRACT

Type 1 diabetes mellitus (T1DM) is one of the most common chronic autoimmune disorders that is typically manifested in early childhood and adolescence, in which pancreatic cells are selectively destroyed by the cells of the immune system. The presence or persistence of some viral infections, like Rotaviruses (RV) in the pancreas, is a major component of the pathogenesis of T1DM. This study was designed to determine the possible role of Rotavirus (RV) in the development of TIDM in Iraqi children and identify its most common prevalent genotypes by genome sequencing. Ninety high-risk children positive for T1DM children and 90 healthy and negative for T1DM were recruited to assess the presence of RV by IgG, reverse transcription-polymerase chain reaction (RT-PCR), and sequencing of the 5' UTR to detect all genotypes of RV. Anti-Glutamic Acid Decarboxylase (anti-GAD) was measured by an ELISA kit. The serum IgG levels showed a significant increase ($3.766 \pm 0.7222$ ng/mL *vs* $0.2144 \pm 0.03175$ng/mL), in patients as compared to controls ($p < 0.0001$). The increasing levels of anti-GAD correlated with the RV infection in most patients but not with healthy controls ($p < 0.01$). RV was detected in children with TIDM but not in the control group, suggesting its contribution as a risk factor for T1DM at 3.5%. Sequencing data revealed that RV G3P8 was the most dominant genotype in this population. This study supports the hypothesis that infection with RV may proportionally play a role in causing TIDM in Iraqi children.

**Keywords:** Type 1 diabetes mellitus, Rotavirus, GAD, RT-PCR, children, Iraq

## INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a T-cell-mediated autoimmune disease in which pancreatic β-cell destruction causes insulin deficiency, leading to hyperglycemia and a tendency to ketoacidosis.[1] According to the International Diabetes Federation (IDF, 2020), there will be around 463 million people living with diabetes globally by 2045, with 5% to 10% diagnosed with T1DM (42.5 to 95 million).[2] T1DM defect related to the immune system destruction of beta-cell antigens and the formation of pro-inflammatory reactions and then contribution of antigen-presenting cells (APCs).[3] In 90% of T1DM cases were positive for glutamic acid decarboxylase (GAD), an enzyme participating in the synthesis of inhibitory aminobutyric acid neurotransmitter in pancreatic islet cells, and an islet cell antigen 2 (IA-2), a tyrosine phosphatase expressed in islet cells.[4] Zinc transporter-8 (ZnT8), the product of the *SLC30A8* gene, is a secretory granule membrane protein of the pancreatic beta cells, which was recently recognized as an autoantigen in T1DM.[5] The human leukocyte antigen (HLA) location on chromosome 6p21.3 is by far the greatest genetic determinant factor of T1DM where HLA-confers susceptibility to at least 50% of the genetic risk to T1DM.[6] These environmental risk factors may be affected by the introduction of autoimmunity or the speed and degree of already continuing beta-cell damage. The possible risk factors may involve early life consumption of cow's milk, postnatal viral infection during pregnancy, older maternal age, and cesarean section delivery.[7] Viral infections have been proposed as one of the etiology of T1DM for many years. Several studies have proposed that certain viruses are associated with autoimmune diabetes in experimental animals.[8] Rotaviruses, responsible for an essential proportion of childhood gastroenteritis, have also been investigated for their connection with T1DM.[9] Rotavirus A (RVA), a genus rotavirus and family member of Reoviridae, is a leading cause of viral diarrhea in children under five years old.[10] Various studies have proposed that certain viruses are associated with the progression of T1DM in animal models.[11] Honeyman and coworkers (2000) reported that diabetes-associated autoantibodies appeared in children concomitantly with a rise in rotavirus IgG antibody titer. Rotavirus also

changes the permeability and cytokine balance in the intestinal mucosa and may thereby enhance autoimmunity. Cytokines released during rotavirus gastroenteritis constitute a significant cause of the severe epithelial dysfunction commonly associated with the disease. Interestingly, T cells are also able to modify intestinal ion secretion in rotavirus gastroenteritis.[12] This study aimed to examine the role of rotaviruses in T1DM patients and identify which dominant genotype is involved in the disease.

## MATERIALS AND METHODS

### Subjects

In this study, 90 children were diagnosed with Type 1 diabetes mellitus from 180 children originally from Baghdad Medical City. Blood was collected from children whose parent(s) gave permission. The blood samples were collected during the period from November 2018 to December 2019. The mean age average of patients and controls was between 1 month and 15 years. A total of 5 mL of blood samples were collected in sterile gel tubes. After signing the informed consent by all individuals participate in this study, information was obtained in a questionnaire about age, gender, immunization, family number, living region, family history of T1DM, and other diseases. The ethical approval was obtained by the Ethics Committee of the Ministry of Health decree number 9382 on 18/12/2018.

### Random Glucose Test

One thousand microliters of the test standard (Human Liqui Color, Germany) and a sample was added to the cuvette, and a one thousand µL of reagent was added to the sample cuvette and the blank cuvette. The cuvette was incubated at 37°C for 5 minutes. The absorbance was measured at 500 nm wavelength against blank. Then glucose concentration was measured according to the equation below, considering that the standard concentration was 100 (mg/dL): glucose = Antibody sample/Antibody standard X standard concentration.

### Anti-GAD Autoantibody Measurement by Enzyme-Linked Immunosorbent Assay (ELISA)

After bringing the temperature of all reagents to 25°C, samples were spun after thawing before starting the assay. An aliquot of 25 µL of sera was dispensed and mixed thoroughly and incubated with a100 µL of reconstituted GAD65-Biotin and SA-POD (G) into each well. Then a peroxidase substrate was added in the dark, and finally, the reaction was stopped, and the absorbance (OD) was measured at 450 nm using a microtiter plate reader (Demeditec, Germany).

### Human Rotavirus IgG (RV IgG) ELISA

Fifty µl of each standard and forty µL of each sample were added to appropriate wells. A total of 10 µL of Antibody of IgG RV was incubated with standard and samples then mixed with a chromogen reagent according to the manufacturer's instructions (Shanghai Yehua Biological Technology, China). The optical density (OD) of each well was read at 4 50 nm.

### RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

According to the manufacturer's instruction, RNA was extracted from blood using a ready-made kit viral nucleic acid extraction kit (Geneaid, Korea). For the detection of RV, RT-PCR was performed using specific primers from the VP7 gene and reagents purchased from AccuPower® RocketScript™ RT. PreMix (BioNeer, Korea).

### Conventional RT-PCR

Oligonucleotide primer of VP7 gene was chosen for amplification of the 5′UTR region, which was used for the detection of RV cDNA fragments by using a conventional PCR using VP7 forward primers (5'-ATGTATGGTATTGAATATACCAC-3') and a reverse primer (5'- AACTTGCCACCATTTTTTCC-3') with an annealing temperature of 54°C to obtain an amplicon size of 885 bp. The amplification conditions started with an initial denaturation of 95°C for 5 min, followed by 40 cycles of denaturation for 30 sec, annealing for 1 min, and extension for 30 sec and final extension for 7 min at 72°C.

### Detection and Genotyping of RV Isolates Obtained from T1DM Patients

A volume of 25µL of amplicons for each sample and 17 pmol from both, forward and reverse primers from VP7 gene of RV for both directions sequencing showed positive for RV by PCR. Amplicons were sequenced in both directions using an ABI Biosystems automated sequencer (Macrogen Company, Korea). Sequence outputs were analyzed for further assurance about the specificity of RV isolates and provide the prevalent RV genotype of RV circulating in T1DM patients in Iraq.

### Sequencing and Bioinformatics Analyses

The sequence data were analyzed using BLAST and Phylip available at the NCBI (www.ncbi.nlm.nih.gov) to determine the RV genome sequence of the amplified region. Phylogenetic tree analysis was carried out using phylip and BioEdit to build a relationship tree comparing the sequences of local isolates with other RV available in the database.

## RESULTS

### Detection of T1DM

Ninety individuals of a total of 180 individuals showed positive results for T1DM. All those individuals presented to the hospitals and medical centers with abdominal pain, vomiting, and diarrhea. The age of individuals ranged from 1-month to 15 years. The glucose levels and age groups were shown in Figure 1. Regardless of the age groups, most patients ended up with the highest levels of glucose in their blood.

Patients were divided into three groups depending on their age (1 month–6 years), (>6–11 years) and 12–15 years, and the glucose levels for each group were measured to show a low level ranging from 45–100, then at moderate levels of 101–150, at high levels of 151-300, and very high levels between 300 to 500 mg/dL (Figure 1). On the other hand, the normal range of the blood glucose levels in the control groups was less than
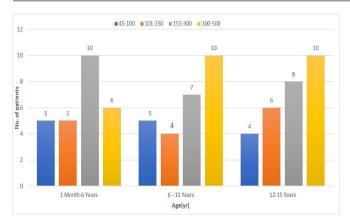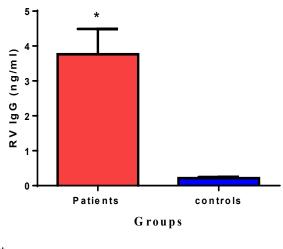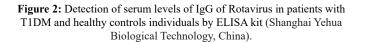
**Figure 1:** In this study, glucose level (in mg/dL) and age groups of patients with TIDM were used. The numbers above the columns represent several subjects with TIDM within each age group.
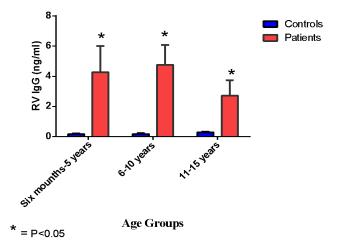


**Figure 2:** Detection of serum levels of IgG of Rotavirus in patients with T1DM and healthy controls individuals by ELISA kit (Shanghai Yehua Biological Technology, China).



**Figure 3:** Age group distribution of serum IgG levels of RV in T1DM patients and controls.

180 mg/dL after two hours of beginning a meal and between 70–130 mg/dL before meals which corresponds well with the American Diabetes Association guidelines (2019).
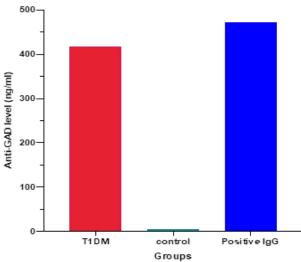
### Detection of Rotavirus IgG by ELISA

The circulating levels of Rotavirus IgG were examined using Rotavirus IgG ELISA kit, and results showed that 28 (31.1%) children, out of a total of 90, were positive for rotavirus IgG. The serum IgG levels showed a significant increase of $3.766 \pm 0.7222$ ng/mL, in patients compared to controls levels of $0.2144 \pm 0.03175$ ng/mL ($p<0.0001$) (Figure 2).

### Detection of Anti-glutamic Acid Decarboxylase (Anti-GAD) Antibodies

The correlation between the level of GAD autoantibody and T1DM in patients and controls showed that these antibodies were higher in patients by 100-fold than individuals without T1DM (Table 3). In addition, the levels of GAD autoantibodies in patients positive for RV IgG showed a further and significant increase ($P<0.01$; Chi-Square $=142.292$) above those observed with individuals who were negative of RV IgG but had T1DM.

### Detection of Rotavirus RNA using Conventional PCR

Five out of 90 patients (5.55%) with T1DM revealed a specific amplification for the presence of RV by detection of VP7 gene
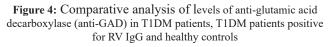


**Figure 4:** Comparative analysis of levels of anti-glutamic acid decarboxylase (anti-GAD) in T1DM patients, T1DM patients positive for RV IgG and healthy controls

**Table 4:** Relationship between anti-GAD antibodies in T1DM patients, T1DM patients' positive of RV IgG compared to apparently healthy controls

|  | *T1DM* | *Control* | *Positive IgG* |
| --- | --- | --- | --- |
| Number of values | 90 | 90 | 28 |
| Minimum | 7.761 | 0.04600 | 7.800 |
| Maximum | 6922 | 20.50 | 2178 |
| Range | 6914 | 20.45 | 2170 |
| Mean | 417.1 | 5.835 | 472.5 |
| Std. Deviation | 967.4 | 2.695 | 714.1 |
| Std. Error of Mean | 102.0 | 0.2841 | 135.0 |

by using a conventional PCR, three of them were very obvious, and the other two showed a faint band (Figure 5).

## DNA Sequencing of Rotavirus Obtained from Children with TIDM

PCR products were cleaned out to eliminate the PCR reagents, and the quality was checked on gel electrophoresis. The cleaned products were used for DNA sequencing of the VP7 gene of RV in both directions by using both forward and reverse primers. Results showed specific sequences of the VP7 gene for RV obtained from T1DM patients (Figure 6). A multiple sequence alignment of the sequence outputs of the local RVs was illustrated in Figure 7. The deduced amino acids of the local RV isolates revealed a change of E (glutamic acid) to D (aspartic acid) at 180 positions when compared to other related RV sequences from the database using BioEdit software (Figure 8). These two amino acids have the same characteristics



**Figure 5:** Detection of RV in children with TIDM by using a conventional PCR. The amplified products run on a 2% agarose gel electrophoresed at 75 V for 1hour and 30 minutes, and stained with ethidium-bromide, then photographed using a smart gel document system (MajorScience, Korea) under a UV transilluminator. Lanes order: M: DNA marker; Lane 22, negative control; lanes 21, 15, and 14 showed specific amplification bands of 885 bp of VP7 RV from three samples obtained from children with TIDM; lanes 11 and 11, weak positive for VP-7 RV; and Lanes 1-10 and 12, 13, 16, 17, 19, 20 were negative for VP7 RV.



**Figure 6:** DNA sequencing profile of the VP7 gene of RV detected in Iraqi children with T1DM
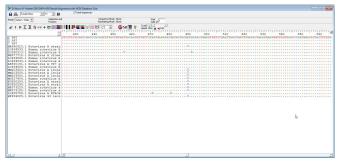


**Figure 7:** Multiple sequence alignment of the VP7 gene of the local Rotaviruses presents in Iraqi children with TIDM by using ClustalX of the NCBI database (www.ncbi.nlm.nih.gov).

(polar and acidic), so this replacement may not significantly influence the resulting protein.

## Phylogenetic Tree Analysis of VP7 Gene of RV

According to the dendrogram phylogenetic tree of the sequences of local RV isolates obtained from Iraqi children with TIDM, one main genotype of RV was identified as Rotavirus G3P8. When these sequences were compared to the currently available NCBI RV sequences of the same genome region, these genotypes were specific to other related NCBI RV sequences (Figure 7). Sequences of the RV local isolates were clustered together very closely, closely with some other NCBI RV subtypes but segregated from others. Hence, sequences of the local isolates (2-vp7, 3-vp7, and 7-vp7) subtypes were clustered closely to subtypes of those derived from the NCBI sequences MH377718.1 (Iraq), LC455759.1 (Thailand), and LC260213.1 (Indonesia), followed by LC434529.1 (Japan), LC434533.1 (Japan), LC434528.1 (Japan), and AB930191.1 (Japan), and segregated apart from MN203563.1, MN203559.1, MN203566.1, MK690523.1 (Czech), MF494825.1, MK873182.1, MG517404.1, KU550295.1, and MK873229.1 (Thailand).



**Figure 8:** Amino acid sequences of the VP7 region of the local RV isolates compared to other closely related isolates revealed replacement of E (glutamic acid) to D (aspartic acid) in the local RV isolates



Note: Branches shorter than 0.0003 are shown as having length 0.0003

**Figure 9:** Dendrogram phylogenetic tree analysis of the VP7 sequences of local isolates of RV (2-vp7, 3-vp7, and 7-vp7) detected in Iraqi children who have TIDM. These sequences were closely related to other NBCBI sequences MH377718.1 (Iraq), LC455759.1 (Thailand), and LC260213.1 (Indonesia), followed by LC434529.1 (Japan), LC434533.1 (Japan), LC434528.1 (Japan), and AB930191.1 (Japan), and segregated apart from MN203563.1, MN203559.1, MN203566.1, MK690523.1 (Czech), MF494825.1, MK873182.1, MG517404.1, KU550295.1, and MK873229.1 (Thailand).

## DISCUSSION

Type 1 diabetes mellitus (T1DM) is one of the most common chronic autoimmune disorders that is typically manifested in early childhood and adolescence, in which pancreatic cells are selectively destroyed by the cells of the immune system (Krzewska and Ben-Skowronek, 2016). Various studies have proposed that certain viruses are associated with the progression of T1DM in animal models[11] Honeyman, and coworkers (2000) reported that diabetes-associated autoantibodies appeared in children concomitantly with a rise in the levels of rotavirus IgG antibody. Rotavirus also changes the permeability and cytokine balance in the intestinal mucosa and may thereby enhance autoimmunity. Cytokines released during rotavirus gastroenteritis are a major cause of the severe epithelial dysfunction commonly associated with the disease. Interestingly, T cells are also able to modify intestinal ion secretion in rotavirus gastroenteritis.[12]

Results of this study showed that infection with RV contributes to T1DM in children by both PCR (5.55%) and ELISA IgG (31.1%), although the size of samples was small. The serum IgG levels of RV showed a significant increase (p <0.0001) in T1DM patients compared to controls. The age group distribution of T1DM patients who were positive for RV had a significantly higher IgG across all age groups (Regnell and Lernmark, 2017). It was previously reported that RV was detected in 28.7%[13] and 25.7%[14] of young children with acute diarrhea in Iraq. It worth noted that local children had received vaccination against RV since 2013,[15] therefore, some IgG higher responses may be due to the injected vaccine rather than an actual infection, and this requires to be qualified by a further follow-up study. This result is still important at a time an effective vaccine is available. Moreover, the absence of a virus in such a large number may be due to viral clearance. Another investigation showed that the risk of T1DM was reduced by 37% among vaccine recipients of pentavalent RotaTeq vaccine than those who received the monovalent Rotarix vaccine (27%).[16]

There was a specific increase in the levels of anti-GAD (*P*<0.01) over those observed with T1DM with no RV. However, its contribution to autoimmunity and beta-cell destruction in not yet understood. It was reported that nearly 60% of individuals who were positive for a single autoantibody may return to seronegativity. The link between GAD autoimmunity and RV infection in TIDM was suggested to be based on an analogy between the glutamic acid decarboxylase (GAD) as one of the main beta cell antigens in T1DM the VP7 major immunogenic protein of RV. Therefore, molecular mimicry with RV promotes autoimmunity to pancreatic islet autoantigens by having sequence similarities with T cell epitopes of tyrosine phosphatase –like insulinoma Ag2 (IA2) and GAD65.[17] Also, RV-VP7, as well as IA-2 and GAD65, can bind to HLA-class II molecules which mediates susceptibility to T1DM. Moreover, RV infection may activate T cells, which can cross-react with 2 protrusive B cell antigens leading to insulitis.[18]

This study showed that the most common subtypes of RV was belong to G3P8 by sequencing and phylogenetic tree analysis in children with T1DM for the first time in Iraq. The protein sequence alignment of the local isolates derived from T1DM patients had amino acid substitution from glutamic acid to aspartic acid. Whether this substitution is only linked to T1DM patients infected with RV is still not clear. These two amino acids have the same characteristics (polar and acidic), so this replacement may not change the resulting protein. Recently in Italy, the first detection of a reassortant G3P[8] RVA was associated with severe acute gastroenteritis among school children.[19]

## CONCLUSION

In conclusion, Although PCR is more sensitive for detecting pathogens, the detectable RV among T1DM children was 5.55% by PCR, but the RV IgG ELISA was much higher (31.1%). This result could be attributed to the spread of RV at lower viral loads or because of the seroconversion after children immunization with RV vaccine introduced in 2013 in Iraq. The increase in IgG was correlated with significantly higher anti-GAD autoantibodies in T1DM children. For the first time in Iraq, the RV G3P8 subtype was shown to be circulating among Iraqi children with T1DM.

## REFERENCES

1. Kahanovitz L, Sluss PM, Russell SJ. Type 1 diabetes–a clinical perspective. Point of care. 2017 Mar;16(1):37.
2. Iqbal A, Novodvorsky P, Heller SR. Recent updates on type 1 diabetes mellitus management for clinicians. Diabetes & metabolism journal. 2018 Feb 1;42(1):3-18.
3. Saberzadeh-Ardestani B, Karamzadeh R, Basiri M, Hajizadeh-Saffar E, Farhadi A, Shapiro AJ, Tahamtani Y, Baharvand H. Type 1 diabetes mellitus: cellular and molecular pathophysiology at a glance. Cell Journal (Yakhteh). 2018;20(3):294-301.
4. Stayoussef M, Benmansour J, Al-Jenaidi FA, Said HB, Rayana CB, Mahjoub T, Almawi WY. Glutamic acid decarboxylase 65 and islet cell antigen 512/IA-2 autoantibodies in relation to human leukocyte antigen class II DR and DQ alleles and haplotypes in type 1 diabetes mellitus. Clinical and Vaccine Immunology. 2011 Jun;18(6):990-993.
5. Gomes KF, Semzezem C, Batista R, Fukui RT, Santos AS, Correia MR, Passos-Bueno MR, da Silva ME. Importance of zinc transporter 8 autoantibody in the diagnosis of type 1 diabetes in Latin Americans. Scientific reports. 2017 Mar 16;7(1):1-7.
6. Groop L, Pociot F. Genetics of diabetes–are we missing the genes or the disease?. Molecular and cellular endocrinology. 2014 Jan 25;382(1):726-739.
7. Awadalla NJ, Hegazy AA, El-Salam A, Elhady M. Environmental factors associated with type 1 diabetes development: a case control study in Egypt. International journal of environmental research and public health. 2017 Jun;14(6):615.
8. Knip M, Simell O. Environmental triggers of type 1 diabetes. Cold Spring Harbor perspectives in medicine. 2012 Jul 1;2(7):a007690.
9. Coppieters KT, Boettler T, von Herrath M. Virus infections in type 1 diabetes. Cold Spring Harbor perspectives in medicine. 2012 Jan 1;2(1):a007682.

10. Damanka SA, Kwofie S, Dennis FE, Lartey BL, Agbemabiese CA, Doan YH, Adiku TK, Katayama K, Enweronu-Laryea CC, Armah GE. Whole genome characterization and evolutionary analysis of OP354-like P [8] Rotavirus A strains isolated from Ghanaian children with diarrhoea. Plos one. 2019 Jun 14;14(6):e0218348.

11. Yeung WC, Rawlinson WD, Craig ME. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. Bmj. 2011 Feb 3;342.

12. Blomqvist M, Juhela S, Erkkilä S, Korhonen S, Simell T, Kupila A, Vaarala O, Simell O, Knip M, Ilonen J. Rotavirus infections and development of diabetes-associated autoantibodies during the first 2 years of life. Clinical & Experimental Immunology. 2002 Jun;128(3):511-515.

13. Salih NE. Rota virus Association diarrhea among children less than 5 years in Mosul city. Tikrit Journal of pure science. 2009;14(3):76-80.

14. Hasan AR, Al-Azzawi DS, Al-Duliami AA, Al-Azawi AK. Anti-rotavirus IgG Seropositivity among Healthy Population and Patients with Acute Diarrhea in Baquba-Diyala Province. Iraqi Journalof Community Medicine. 2011;24(2).: 200-204.

15. American Diabetes Association. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes—2019. Diabetes Care, 2019;42(Supplement 1), S13-S28.

16. Harrison LC, Perrett KP, Jachno K, Nolan TM, Honeyman MC. Does rotavirus turn on type 1 diabetes?. PLoS pathogens. 2019 Oct 10;15(10):e1007965. doi: 10.1371/journal.ppat.1007965.

17. Honeyman MC, Stone NL, Harrison LC. T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA-2: potential for mimicry with rotavirus and other environmental agents. Molecular medicine. 1998 Apr;4(4):231-239. https://doi.org/10.1007/bf03401920

18. Ballotti S, de Martino M. Rotavirus infections and development of type 1 diabetes: an evasive conundrum. Journal of pediatric gastroenterology and nutrition. 2007 Aug 1;45(2):147-156. https://doi.org/10.1097/MPG.0b013e31805fc256

19. Esposito S, Camilloni B, Bianchini S, Ianiro G, Polinori I, Farinelli E, Monini M, Principi N. First detection of a reassortant G3P [8] rotavirus A strain in Italy: a case report in an 8-year-old child. Virology journal. 2019 Dec;16(1):1-7.. https://doi.org/10.1186/s12985-019-1173-1.