Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ DERGİSİ

Cukurova Med J 2016;41(3):533-538
DOI: 10.17826/cukmedj.237508

ARAŞTIRMA/RESEARCH

Detection of norovirus infections in Canakkale with ELISA and RT-PCR

Norovirüs enfeksiyonlarının Çanakkale'de ELISA ve RT-PCR ile saptanması

Arif Aksu[1], Alper Akçalı[2*]

[1]Canakkale State Hospital, Çanakkale, Turkey
[2]Canakkale Onsekiz Mart University School of Medicine, Department of Medical Microbiology, Çanakkale, Turkey

Cukurova Medical Journal 2016;41(3):533-538.

Abstract

Purpose: Noroviruses are important agents of gastroenteritis worldwide in every age group. There is no large countrywide dataset available for norovirus infections in Turkey. This is the first investigation of noroviruses in Çanakkale, and we aimed to compare commercial ELISA and real time RT-PCR assays in clinical use.

Material and Methods: Fecal samples from 92 clinical gastroenteritis cases were collected and stored at -20°C. Norovirus antigen was investigated using RidaScreen ELISA and norovirus GI and GII molecular detection made with RealStar Norovirus RT-PCR kit 2.0.

Results: We found that 16 (17.4%) samples were positive for norovirus in our study group. Using ELISA, 10 samples were positive from 92 samples. In PCR analysis, internal control amplification failed for four samples. We found 15 of 88 samples positive for real time RT-PCR analysis; of these one was GI and 14 were GII. When we accepted PCR as the reference test, the sensitivity and specificity of ELISA were calculated as 60% and 98% respectively.

Conclusion: Single cases of norovirus infection can be seen even in rural cities like Çanakkale. There is a need to set up diagnostic capabilities for norovirus infections. ELISA has a low sensitivity, but this method is cheaper than RT-PCR and is not affected by PCR inhibitors.

Key words: norovirus, gastroenteritis, ELISA, polymerase chain reaction

Öz

Amaç: Norovirüsler dünya genelinde her yaş grubunda önemli ishal etkenleridir. Türkiye'de norovirüs enfeksiyonları ile ilgili ülke genelinde geniş bir veri bulunmamaktadır. Bu çalışma Çanakkale'deki norovirüslerle ilgili ilk araştırma olup, tanıda ELISA ve real time RT-PCR testlerinin klinik kullanımlarının değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Klinik olarak ishali olan 92 vakadan dışkı örnekleri toplanmış olup çalışmaya kadar -20C'de saklanmıştır. Norovirüs antijeni RidaScreen ELISA kiti, GI ve GII moleküler saptaması RealStar Norovirüs RT-PCR kiti ile yapılmıştır.

Bulgular: Çalışmada 16 (%17,4) örnek norovirüs için pozitif bulunmuştur. ELISA ile 92 örnekten 10 tanesi pozitiftir. PCR çalışmasında 4 örnekte internal kontrol çalışmamış olup, 88 örnekten 15 adedinde RT-PCR'de pozitiflik saptanmıştır. Bunlardan biri GI ve ondördü GII tespit edilmiştir. PCR'ı referans test olarak aldığımızda ELISA'nın duyarlılığı %60, özgüllüğü %98 bulunmuştur.

Sonuç: Çanakkale gibi bir ilde bile norovirüs infeksiyonları birer vaka olarak bile görülebilir. Norovirüs infeksiyonlarının tanısı için altyapı oluşturulmalıdır. ELISA düşük bir duyarlılığa sahip olmakla birlikte RT-PCR'a göre ucuz olup, PCR inhibitörlerinden etkilenmemektedir.

Anahtar kelimeler: Norovirus, ishal, ELISA, polimeraz zincirleme reaksiyonu

INTRODUCTION

Noroviruses are positive-sense single-stranded RNA viruses and members of a single genus of the Caliciviridae family. The norovirus genus shows high genetic variability. Classification of the norovirus genus is based on the sequence diversity in the ORF2-encoded VP1 protein. There are five recognized genogroups (GI-GV)[1].

Currently over 30 genotypes or genetic clusters have been defined within these genogroups. Genogroup II noroviruses are more common than those of genogroup I[2]. Genotype 4 (GII.4) of

Yazışma Adresi/Address for Correspondence: Dr. Alper Akçalı, Canakkale Onsekiz Mart University, School of Medicine, Department of Medical Microbiology, E-mail: aakcali@isnet.net.tr
Geliş tarihi/Received: 23.02.2016 Kabul tarihi/Accepted: 27.03.2016

genogroup II is responsible for most infections globally. Moreover, GII.4 strains are also mostly associated with outbreaks[3].

Norovirus infections can be seen in persons of all ages. Generally, clinical infection has a 24–48 h incubation period. Acute onset of nausea, vomiting, abdominal cramps, myalgias, and non-bloody diarrhea is common. Vomiting and non-bloody diarrhea can be severe symptoms of norovirus illness. Symptoms usually resolve in 2–3 days. However, recent studies report that symptoms can last 4-6 days in patients affected during hospital outbreaks and in children younger than 11 years of age[4].

Noroviruses were unknown in Turkey before the first outbreak was reported from central Anatolia in 20085. Since then, limited reports have been published on norovirus infections in Turkey. The National Reference Laboratory for Public Health reported data from 11 different cities for the year 2009. From 147 samples, 57 (38.7%) were positive for noroviruses[6]. In their study, 17 samples were from Canakkale and all of them were negative for noroviruses. In several other publications from Turkey, 8.1% to 17% norovirus-positive cases were reported from non-outbreak investigations[7,8]. There is no large countrywide dataset available for norovirus epidemiology and to date, no algorithm for laboratory diagnosis has been widely accepted in Turkey.

Noroviruses were first identified using immunoelectron microscopy. This method has now limited value in clinical use because of its low sensitivity of 15%[9]. Immunological tests were developed to detect the antigen of noroviruses in feces. There are mostly ELISA based methods manufactured; for example RIDASCREEN (R-Biopharm, Darmstadt, Germany) and IDEIA/Prospect (Oxoid, Hampshire, UK). Immunochromatographic assays were introduced into the market by several manufacturers. All these immunological antigen detection methods have varying sensitivity in clinical use, such as 49.5 and 76%. If the patient is infected with GI, the sensitivity can be as low as 8-15%. The first cloning of the Norwalk virus[10] was successful in 1990 and further RT-PCR assays have been developed. Diagnostic methods for norovirus infections were improved in the last decade. Now there are many commercial molecular tests available on the market. Because extensive variations among noroviruses occur because of an antigenic drift or untranslated mutation, serological or molecular assays can fail to detect noroviruses. Thus diagnostic methods were compared in the literature to each other in all clinical situations.

In this study, we aimed to investigate the presence of norovirus in gastroenteritis cases in Canakkale, Turkey and to compare ELISA and PCR to find which method can be useful for diagnosis in a rural city like ours.

MATERIALS AND METHODS

Sample collection

Samples without any other infectious agents were collected between September 2012 and August 2013 from patients admitted with gastroenteritis and watery diarrhea to the Çanakkale Onsekiz Mart University School of Medicine Hospital. The samples were stored at -20°C until laboratory analysis A total of 92 fecal samples were included in this study. Our study was approved by the Clinical Research Ethical Committee of Çanakkale Onsekiz Mart University. No: 2012/ 050-99-144.

Laboratory analysis

Norovirus antigens were investigated using RIDASCREEN Norovirus Antigen ELISA kit (R-Biopharm, Darmstadt, Germany.) Tests were done according to the manufacturer's instructions, using a Biotek ELx50 microplate washer and an ELx800 microplate reader (Winooski, VT, USA) . Cut-off was determined by adding 0.150 to the optical density (O.D) of negative control, supplied by the kit. Samples with O.D. above the cut-off level were accepted as positive for the norovirus antigen.

Norovirus RNA detection was performed using the RealStar Norovirus RT-PCR kit 2.0 (altona Diagnostics GmbH, Hamburg, Germany.) This kit includes three different probes; Cy5 dye for norovirus GI, FAM for norovirus GII and JOE dye for internal control detection. After the samples were thawed, the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) was used for RNA extraction. Internal controls (IC) supplied by the RT-PCR kit were included in each sample during the extraction step as an amplification control. Five µl of extraction product was used for real time RT-PCR reaction. Each PCR run included nuclease-free water as a negative control without any template to

determine the presence of amplicone contamination. Rotor Gene Q RT-PCR (Qiagen, Hilden, Germany) was used for the real-time PCR reaction and detection steps. Samples that failed IC amplification were excluded from the sensitivity and specificity analysis.

Statistical analysis was done using SPSS 19.0. We performed statistical evaluation with a Chi-square test. Associations were considered significant at a p value of less than 0.05.

RESULTS

We collected 92 samples from patients; 62% were male and %38 were female. The mean age of the patients was 30.1± 25.2 (min 1 max 85) years. We performed ELISA and RT-PCR on the 92 samples. In the RT-PCR analysis, internal control (IC) amplification failed for four samples. ELISA results were negative for these four samples. These samples were not included for performance comparison between ELISA and PCR.

According to the ELISA, 10 samples were positive from 92 samples. We found 15 of 88 samples positive in the RT-PCR analysis; of these, one was GI and 14 were GII.-When we accepted RT-PCR as the reference test, the sensitivity and specificity of ELISA were calculated as 60% and 98% respectively (Table 1).

There was no statistically significant difference between norovirus positive and negative groups for age and gender. Most positive samples were from autumn (Table 2). Because the sample size was small we did not make a statistical interpretation.

Table 1. Detection of norovirus by ELISA (RidaScreen) and real time RT-PCR (RealStar)

	PCR Positive	PCR Negative	Total
ELISA Positive	9	1	10
ELISA Negative	6	72	78
Total	15	73	88

Sensitivity of RidaScreen ELISA; 60% (9/15)
Specificity of RidaScreen ELISA; %98 (72/73)

Table 2. Seasonal distribution of norovirus positive samples.

Month	Total sample	Positive*
January	28	2
May	11	3
June	9	1
September	9	4
October	4	1
November	15	4
December	2	1

* Positive with one of the methods used
If none of the samples were found positive in a month, it is not presented.

DISCUSSION

We found 16 (17.4%) samples were positive for norovirus in our study group. ELISA had a sensitivity of 60%. Most of the cases (93%) had GII, and only one sample was found positive for GI by RT-PCR. It is well known that PCR inhibitors are common in fecal samples. IC amplification by RT-PCR failed for four samples in our study. In such situations, tests can be redone, but this will duplicate the cost of diagnosis.

Patel et al. estimated with their literature review, that each year NoVs cause 64,000 episodes of diarrhea requiring hospitalization and 900,000 clinic visits among children in industrialized countries, and up to 200,000 deaths of children under five years of age in developing countries[11].

Now that the rotavirus vaccine has become available and has started to be used, it seems norovirus may be the most important agent for viral gastroenteritis. The first norovirus outbreak reported in Turkey was in 2008[5]. This outbreak affected four cities in Anatolia. Uyar et al. reported 61.5% sensitivity and 100% specificity for RidaScreen ELISA when compared to in-house real-time RT-PCR during this outbreak. They found 13 samples positive for RT-

PCR; nine samples were GI and four samples were GII. Altindis et al. reported in their study data from samples collected in the period 2006-20078. We can accept their published study as the first one from a clinical non-outbreak investigation in Turkey. They reported 17% norovirus positivity.

Another study including data from Canakkale and 10 other cities reported 38.7% norovirus cases (57 from 147 samples), but there was no sample from Canakkale positive for noroviruses[6]. Özkul et al. reported 15.1% positivity for noroviruses in their study group in İstanbul[12]. From an outbreak investigation that affected 7800 persons in Tokat, Turkey Gonen reported norovirus infections[13]. During investigation of this outbreak, 24 samples were collected. From these, 11 were positive for noroviruses. Col et al. reported 9.6% norovirus positivity from Istanbul[14]. In another study from İstanbul, Inan et al. reported 8.1% positivity[7].

There are three other reports from Ankara reporting 10% (15/150)[15], 14.1% (141/1000)[16] and 16% (8/50)[17] positive results for noroviruses. Interestingly, during an outbreak in 2009 among military personnel in a USA air-base in İncirlik, Turkey, 16 samples from 37 samples were found positive for GII norovirus[18]. The sequence similarity of these noroviruses was distinct from previously reported sequences from Turkey, thus this outbreak seems to have been an importation. These are all the data available for Turkey about norovirus infections. Our data is the first from Canakkale, and the positivity rate is similar to others from Turkey. These data show that noroviruses are an important cause of gastroenteritis in Turkey. There is a potential risk for outbreaks, and furthermore, single cases can be seen even in rural cities like Çanakkale.

Morillo et al. reported overall 61.8 % sensitivity and 92.5% specificity and concluded an outbreak sensitivity of 87.9% and specificity of 83.8% for the RidaScreen ELISA kit[19]. Rovida et al. reported 49% sensitivity and 93.3% specificity for RidaScreen ELISA[20]. Bruins et al. investigated RidaQuick immunochromatographic point of care (PoC) test, and reported 57.1% sensitivity and 99.1% specificity[21]. On the other hand, Bruggink et al. from Australia reported 83% sensitivity and 100% specificity for the RidaQuick immunochromatographic assay[22]. Interestingly, they concluded that freezing and thawing the samples had improved the sensitivity. In another study, Bruggink et al. reported 62% sensitivity and 98.6% specificity for another manufacturer's immunochromatographic kit, Standard Diagnostics (Korea), Bioline[23]. Kele et al. from Hungary investigated the use of IDEIA norovirus ELISA (DakoCytomation, UK) kit, and reported sensitivity as 78.9% and specificity as 100%[24]. But Kirby et al. reported 45% sensitivity in their study for the IDEA assay[25]. The major drawback of immunological assays is the poor detection rate of GI noroviruses; in most publications these assays failed to detect GI-positive samples. In our study, the only GI RT-PCR positive sample was positive for ELISA too. As in some other studies, we found a low sensitivity for the ELISA method. But ELISA is cheaper than RT-PCR and is not affected by PCR inhibitors. Performing ELISA does not require an extraction step or complex equipment.

In last decade, RT-PCR assays for noroviruses have become the gold standard[26]. But, they require expensive equipment and the cost of commercial PCR tests can be high. Currently in Turkey it is not possible to have RT-PCR systems in every city, but samples can be transported to neighboring centers. Duizer et al. suggested using a minimum of six samples for outbreak confirmation when ELISA is used[27].

If the laboratory methods are not available, the Kaplan criteria can be used for identifying noroviruses as possible source of an outbreak. In 1982, Kaplan et al. determined that the presence of four features can be useful for predicting norovirus as the causative agent; (1) vomiting in more than 50% affected persons; (2) mean (or median) incubation period of 24–48 h; (3) mean (or median) duration of illness of 12–60 h; and (4) negative stool culture result for bacterial pathogens[28]. In some cases, a definite diagnosis is essential. If the affected person is a health care provider or a food handler, they can cause outbreaks when they return to their work[28]. If ELISA or PoC is available, it can be used for first line diagnosis in situations like this. If the test is negative and no other microbiological agent can be shown, RT-PCR assays can be requested.

A limitation of our study is the small size of the sample group. However, we did not plan this work as a surveillance study of our city and we had limited resources. Surprisingly we found 16 (17.4%) samples positive with one of the methods used. This shows that norovirus is an important agent for gastroenteritis in our region. The sampling method used in our study may be not suitable for a

comprehensive epidemiological interpretation, but our work is the first reporting noroviruses from our city, and has shown a significantly high positivity rate.

Çanakkale is a rural city, but it is a crossroads between Europe and the Aegean part of Turkey. Travelers from eastern Europe pass through our city, and cruise ships sometimes come to Çanakkale. Our city could be an importation or exportation point of norovirus infections. Further molecular studies are needed to investigate the genetic relationship of norovirus strains in our region to track the sources of the viruses. Using RT-PCR in diagnostics will help in genotyping noroviruses.

Noroviruses can be found in rural places in Turkey. Both methods, ELISA and RT-PCR, have several advantages for norovirus detection. ELISA is simple to perform and cheap, but has a low sensitivity. RT-PCR has high sensitivity, but routine diagnostics with RT-PCR may be not cost-effective in rural places. Immunochromatographic assays show promise for simple and fast diagnosis in future; therefore, further studies must include the clinical evaluation of these tests for clinical use in Turkey. If definite diagnostics for a single patient are necessary, RT-PCR must be performed. Health care centers in Turkey must keep in mind that norovirus cases can occur in their facilities and must be ready for diagnostic support.

Acknowledgements

This study was supported by Çanakkale Onsekiz Mart University Scientific Research Support Department, No:2012/031.

REFERENCES

1. Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS. Norovirus classification and proposed strain nomenclature. Virology. 2006;346:312–23.
2. Kirby A, Turriza-Gómara MI. Norovirus diagnostics : options, applications and interpretations. Expert Rev Anti-infective Ther. 2012;10:423–33.
3. Hoa Tran TN, Trainor E, Nakagomi T, Cunliffe NA, Nakagomi O. Molecular epidemiology of noroviruses associated with acute sporadic gastroenteritis in children: Global distribution of genogroups, genotypes and GII.4 variants. J Clin Virol. 2013;56:185–93.
4. Patel MM, Hall AJ, Vinjé J, Umesh DP. Noroviruses: a comprehensive review. J Clin Virol. 2009;44:1–8.
5. Uyar Y, Carhan A, Ozkaya E, Ertek M. Evaluation of laboratory diagnosis of the first norovirus outbreak in Turkey in 2008. Mikrobiyol Bul. 2008;42:607–15.
6. Albayrak N, Yagci-Caglayik D, Altaş AB, Korukluoglu G, Ertek M. Refik Saydam Hıfzıssıhha Merkezi Başkanlığı, Viroloji Referans ve Araştırma Laboratuvarı, 2009 yılı akut viral gastroenterit verilerinin değerlendirilmesi. Turk Hij ve Deney Biyol Derg. 2011;68:9–15.
7. Inan N, Kabakoğlu Ünsür E, Demirel A, Mamçu D, Sönmez E et al. Akut viral gastroenterit öntanılı vakalarda rotavirus, adenovirus ve norovirus sıklığının araştırılması. Ankem Derg. 2014;28:14–19.
8. Altindis M, Bányai K, Kalayci R, Gulamber C, Koken R, Yoldas Y et al. Frequency of norovirus in stool samples from hospitalized children due to acute gastroenteritis in Anatolia, Turkey, 2006-2007. Scand J Infect Dis. 2009;41:685–88.
9. Kapikian AZ. The discovery of the 27-nm Norwalk virus: an historic perspective. J Infect Dis. 2000;181 Suppl:S295–302.
10. Xi JN, Graham DY, Wang KN, Estes MK. Norwalk virus genome cloning and characterization. Science. 1990;250:1580–83.
11. Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinje J, Parashar UD. Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emer Infect Dis. 2008;14:1224–31.
12. Ozkul AA, Kocazeybek BS, Turan N, Reuter G, Bostan K, Yılmaz A et al. Frequency and phylogeny of norovirus in diarrheic children in Istanbul, Turkey J Clin Virol. 2011;51:160–64.
13. Gönen İ. Management of a large outbreak caused by norovirus and Campylobacter jejuni occurred in a rural area in Turkey. Nobel Med. 2013;9:47–51.
14. Cöl D, Biçer S, Ciler Erdağ G, Giray T, Gürol Y, Yılmaz G et al. Annual report on norovirus in children with acute gastroenteritis in 2009 and their genotypes in Turkey. Infez Med. 2013;214:261–69.
15. Mitui MT, Bozdayi G, Ahmed S, Matsumoto T, Nishizono A, Ahmed K. Detection and molecular characterization of diarrhea causing viruses in single and mixed infections in children: A comparative study between Bangladesh and Turkey. J Med Virol. 2014;86:1159–68.
16. Altay A, Bozdayı G, Meral M, Dallar Bilge Y, Dalgıç B, Özkan S et al. Investigation of norovirus infection incidence among 0-5 years old children with acute gastroenteritis admitted to two different hospitals in Ankara, Turkey. Mikrobiyol Bul. 2013;47:98–108.
17. Akhter S, Türegün B, Kiyan M, Gerçeker D, Guriz H, Şahin F. Investigation of seven different RNA viruses associated with gastroenteritis in children under five years old. Mikrobiyol Bul. 2014;48:233–41.
18. Ahmed SF, Klena JD, Mostafa M, Dogantemur J,

Middleton T, Hanson J et al. Viral gastroenteritis associated with genogroup II norovirus among U.S. military personnel in Turkey, 2009. PLoS One. 2012;7:e35791.
19. Morillo SG, Luchs A, Cilli A, Ribeiro CD, Calux SJ, Carmona Rde C et al. Norovirus 3rd Generation kit: An improvement for rapid diagnosis of sporadic gastroenteritis cases and valuable for outbreak detection. J Virol Methods. 2011;173:13–6.
20. Rovida F, Campanini G, Sarasini A, Adzasehoun KM, Piralla A, Baldanti F. Comparison of immunologic and molecular assays for the diagnosis of gastrointestinal viral infections. Diagn Microbiol Infect Dis. 2013;75:110–1.
21. Bruins MJ, Wolfhagen MJHM, Schirm J, Ruijs GJ. Evaluation of a rapid immunochromatographic test for the detection of norovirus in stool samples. Eur J Clin Microbiol Infect Dis. 2010;29:741–3.
22. Bruggink LD, Witlox KJ, Sameer R, Catton MG, Marshall JA. Evaluation of the RIDA(R)QUICK immunochromatographic norovirus detection assay using specimens from Australian gastroenteritis incidents. J Virol Methods. 2011;173:121–6.
23. Bruggink LD, Catton MG, Marshall JA. Evaluation of the Bioline Standard Diagnostics SD immunochromatographic norovirus detection kit using fecal specimens from Australian gastroenteritis incidents. Diagn Microbiol Infect Dis. 2013;76:147–52.
24. Kele B, Lengyel G, Deak J. Comparison of an ELISA and two reverse transcription polymerase chain reaction methods for norovirus detection. Diagn Microbiol Infect Dis. 2011;70:475–8.
25. Kirby A, Gurgel RQ, Dove W, Vieria SC, Cunliffe NA, Cuevas LE. An evaluation of the RIDASCREEN and IDEIA enzyme immunoassays and the RIDAQUICK immunochromatographic test for the detection of norovirus in faecal specimens. J Clin Virol. 2010;49:254–7.
26. Mattison K, Grudeski E, Auk B, Brassard J, Charest H, Dust K et al. Analytical performance of norovirus real-time RT-PCR detection protocols in Canadian laboratories. J Clin Virol. 2011;50:109–13.
27. Duizer E, Pielaat A, Vennema H, Kroneman A, Koopmans M. Probabilities in norovirus outbreak diagnosis. J Clin Virol. 2007;40:38–42.
28. Kaplan JE, Gary GW, Baron RC, Singh N, Schonberger LB, Feldman R et al. Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. Ann Intern Med. 1982;96:756–61