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USING OF BROADLY REACTIVE PRIMER PAIR IN RT-PCR DETECTION OF SMALL ROUND-STRUCTURED VIRUSES (SRSVs)

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Abstract

Small round-structured viruses (SRSVs) are causative agents of non-bacterial acute gastroenteritis at humans. They are members of family caliciviridae which are similar to picornaviruses in size (28-30 nm) and specific by cup-shaped outline on their surface. The virions contains single-stranded , positive-sense RNA(7.4 – 7,7 kb) and have not the envelope.Actualy, SRSVs are members of caliciviridae NLVs genus (Norwalk like viruses). SRSV strains are: NV-8FIIa/68/US; KY-89/89/9; Sa-1283/84/J; SMA/76/US; 925/92/UK; OTH-25/89/9.The actual SRSVs oligonucleotide detection primers are signed as 36, 35, 69, 78 etc. and for which is known the 5` position. There are also oligonucleotide primers for which precise location within genome is not yet known (primer signed as 86, 95, 96 etc.). Obtained results presented in this paper showed high sensitivity and specifity of molecular SRSVs detection from stool specimens of clinical patients with specific clinical symptoms of acute gastroenteritis, by using of broadly reactive oligonucleotide primers (Ni and E3) in RT-PCR method of amplification and detection by transilluminator after separation in agarose gel electrophoresis.

Key words: SRSVs; gastroenteritis; RT-PCR

Introduction

The first report about non-bacterial acute gastroenteritis and their viral etiology, is dated from place Norwalk (Ohio- USA). In a primary school were infected teachers, staff members and children and it was determined viral causative agent named Norwalk virus (NV). Several another viruses which caused gastroenteritis around the world, were named according to geographical region or place were are detected (Snow Mountain virus, Southampton virus, Hawaii virus, Sapporo virus etc.) (Adler and Zickl., 1969; Kapikian et al., 1972.). These Norwalk – like are named Small round – structured viruses (SRSVs). They are members of family Caliciviridae. The virions contains single – stranded positive sense RNA. The 5` of viral RNA coded non – structural proteins, but 3` end is oposite to viral RNK (7.3 – 7.6 kb) contain three main ORF. According to nucleotide positions is performed molecular differentiation of two Caliciviridae genuses:

1. Norwalk – like viruses (NLVs) including SRSVs.
2. Sapporo – like viruses (SLVs).

The transmition way is faecal/oral, close human contact or contaminated Food or water (Jiang et al., 1993.; Liu et al., 1995.).

These viruses cannot be cultivated in cell or organ culture or in a practical animal model and in the first time, method for characterization each newly identified agent has been lacking. Some of them were characterized antigenically by immune electron microscopy (IEM) and cross-chalenge studies. For IEM and direct EM detection of SRSVs, it is necessary at least 10 / ml of viral particules in clinical specimen. Recently, RT-PCR method is most sensitive for detection and characterisation of viruses from NLV and SLV genus, as well (Caul and Appleton, 1982.; Jiang et al., 1992.).

NV genome was cloned and characterized as a single – stranded RNA genome of about 7,7 kb which encode three open reading frames (ORFs). The longest ORF1 encode 1.738 amino acid polypeptide which has similarity to the polyprotein of picornaviruses. ORF2 encodes capsid protein while ORF3 is responsible for synthesis of 22kDa polypeptide with unknown function. The genomic organisation of NV is similar to another SRSVs an caliciviruses such as Rabbit hemorrhagic disease virus (RHDV). The RNA polymerase region of SRSVs showed relatively stability. Oligonucleotide primers constructed for highly conserved regions of caliciviruses and SRSVs have been used in RT – PCR assays for detecting SRSVs in stool specimens collected from infected individuals. In conjuction with DNA sequencing, it is possible the comparison of partial genomic sequences of SRSVs from different geographical areas. Because of the extensive genomic diversity most RT-PCRs can detect only small proportion of SRSV strains (Gray et al., 1997.; Lambden et al., 1993.; Appleton, 1987.).

In this study we used broadly reactive oligonucleotide primers for detection of SRSVs associated with appearance of acute gastroenteritis.

Material and methods

Viral SRSV-RNA was extracted from 100μl of 10% faeces extract (BSS – 199 balanced salt solution) taken from 40 clinical patients, by " Boom " method (Gua – Nidinium isothiocyanate silica gel extraction or GTC), eluted in 20μl of Rnase – free water containing of 20 units ribonuclease inhibitor (Rnasin).

Step of viral RNA reverse transcription to corresponding cDNA was performed by using of 1μl random primer (PdN6- Pharmacia Biotech). After heating at 70ºC (5 min.), chilling on ice and adding of 10 μl RT-reaction mix (10x buffer II, 50mM MgCl , dNTPs, M-MLV-RT, Rnase – free destiled water). SRSV-RNA is

producted by incubation for 10 min. at room temperature, followed by incubation at 37ºC for one hour and final reaction termination(95ºC for 5 min., chilling on ice).

Five microlitres of producted DNA is used with 45 μl reaction mix (10mM Tris-HCl, pH-8,3; 50 mM KCl; 2mM MgCl ; 350 μM each dNTP, 1 unit Taq polymerase and 20 pmoles final concentration of Ni and E3 primers (Green et al., 1995, Gray et al., 1997.).

Sequence of Ni primer:

5' – GAATTCCATCGCCCACTGGCT - 3'

Sequence of E3 primer:

5' – ATCTCATCATCACCATA - 3'

For amplification of SRSV-target nucleotide sequences it was used the following SRSV-RT-PCR programme (PCR-engine, Perkin Elmer 2400):

94ºC – 2 min. (x1)

95ºC – 1min.

40ºC – 1 min. (x30)

72ºC – 1 min. and 5 min.
15ºC - hold

The detection of specific PCR amplification products (113 bp) was detected by electrophoretic agarose gel separation (containing ethidium bromide) and visualisation by UV transilluminator. We used the following PCR markers : 50, 150, 300, 500, 750 and 1000 bp (Promega corporation). We used also positive SRSV specimen as positive control and Rnase free dH O as negative control(Green et al., 1993.; Green et al., 1995.; Gray et al., 1997.; Boom et al., 1990.).

Results

After guanidinium isotiocyanate GTC/silica gel extraction of SRSV-RNA from 40 faeces specimens of clinical patients and molecular amplification of SRSV poly-merase RNA gene sequences by using of RT-PCR method in Perkin Elmer PCR engine 2400 and scaning of gel after electrophoretic separaton of PCR products by UV transilluminator, we detected specific 113 bp PCR products for specimens number 7 and 8 (first group patients – Picture – 1.) and specimens 4,5,7,8,11,16,18

and 19 (second group patients- Picture –2.). Among them clear positive were specimens 5,7,8 and 16, but mild positive all other specimens. In the second group it was characteristic non-specific amplification for specimens 3,6,7,9,10,14,15 and 19. Positive control was clear (lane + in first picture and lane 21 in the second picture) and as well valid negative control (Rnase free destiled water)

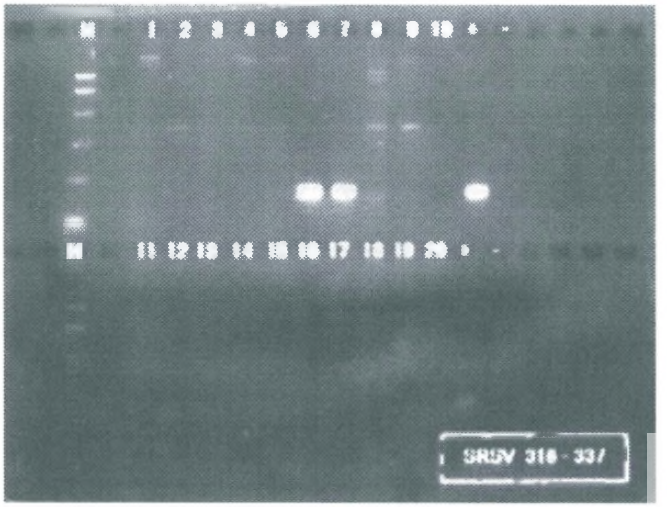

Figure 1. – Electrophoretic separation of PCR-SRSV amplification products in 3% NuSieve agarose gel containing 0,5 μg/ml of ethidium bromide. PCR markers: 50, 150, 300, 500, 750 and 1000 bp (Promega Corporation). Under UV illumination (transilluminator) specimens 6 and 7 were clear positive, but 8 and 9 were mild positive (113 bp) while other specimens were negative. + - positive control; - negative control.

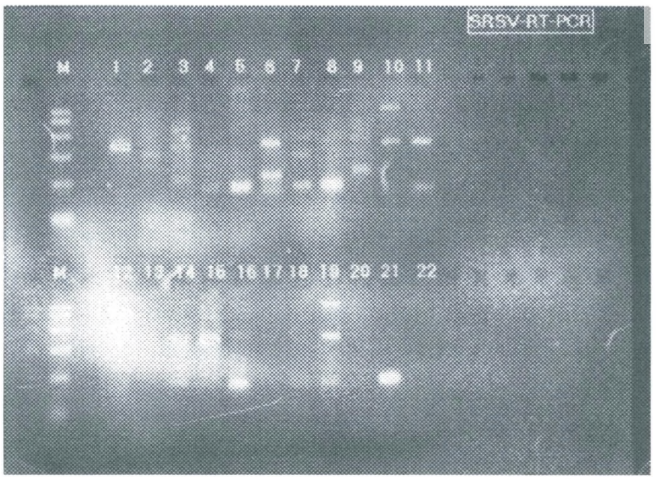

Figure 2. – Electrophoretic separation of PCR-SRSV amplification products in 3% NuSieve agarose gel (containing 0,5 μg/ml ethidium bromide). Positive specimens were number4, 5,6,7,8,11,14,16,18 and 19 (clear positive –5,7,8,11 and 16 while others were mild positive). Clear negative specimens were 12,13,17 and 20. All others have had non-specific amplification bands (specimens number 2, 3, 6, 7, 9, 10, 11, 14,15,16,18 and 19).Lane 21- positive control; lane 22- negative control (Rnase free destiled water). M – PCR markers: 50,150,300,500,750 and 1000 bp (Promega Corporation – USA).

Discussion

Our investigations of stool specimens by SRSV-RT-PCR method showed great sensitivity but great level of non-specific PCR amplification as well (more than 50% of analysed specimens), despite very precise handling during experimental work and high quality of positive and negative control bands obtained by electrophoretic separation in agarose gel. It is clear visible on electrophoregrams (Figure 1 and 2).

According to many published data regarding RT-PCR of SRSVs in the NLVs group, three set of oligonucleotide primers were designed to highly conserved sequences of NV genome:

Primer pair 36/35 – highly conserved to RNA polymerase region of NV genome (PCR product – 470 bp).

Primer pair 39/69 – nested set of primers located within 36/35 region of NV genome (PCR product -158 bp).

Primer pair 78/80 – location in the non-structural 2C region of NV genome (PCR product – 289 bp).

It was found that 69/39 primer set detect more positive samples than primer set 36/35. The smaller PCR products (158bp) from primer set 69/39 also resulted in higher sensitivity of SRSVs detection (Gray et al., 1997.; Poljsak-Prijatelj et al., 2001.; Jiang et al., 1990.; Jiang et al., 1993.).

In our investigation we used broadly reactive primers pair Ni/E3. Ni primer is actually recently signed primer number 81 in primer group specific for detection of viruses of NLVs genus (including SRSVs). E3 is specific for SLVs genus of Caliciviridae family. Because of high level of non-specific PCR amplification, it is necessary to perform DNA sequencing as final checking and analysing. However, RT-PCR combined with nucleotide sequence analysis is powerful approach to study of sequence variation in many virus families. The usefulness of this way in studying of the genetic diversity of SRSVs in NLVs group is already demonstrated recently particularly because of their cultivation impossibility in either, cell culture or animal models. SRSVs sequence database is very important for understanding of their epidemiology and designing of most optimal primers for virus detection and characterization by RT-PCR, and decreasing of non-specific PCR amplification which was characteristic in our molecular biological experiments (Blacklow and Greeenberg, 1991.; Wang et al., 1994.).

The viruses particularly those with RNA genomes, showes more nucleotide sequence variability than DNA viruses and this fact may be a considerable problem regarding primer-target sequences binding. Actually, such primer can cause non-specific amplification of non-target DNA sequences and also can match two distanly related viruses or some variants of corresponding virus type. Appearance of non-target hybrid DNA is probably the main limiting factor about the yield of PCR amplified target products. PCR primers should normally have a minimum 17 or 18 bases and maximum 28 bases. Shorter or longer sequences can lead to their non-specific annealing at temperatures usually used for PCR. The primer binding to target DNA sequence depends on G + C content, which must be at least 45%. It is clear that G + C rich primers will have less ability to hybridize with human genomic DNA which has a high A + T content (more than 60%). Under PCR conditions, two primer sequences can make, so called, primer – dimmer formations (fused primer sequences capable for rapid replication in PCR conditions). However, all above mentioned factors can cause non-specific amplifications and appearance of more electrophoretic bands in the gel, visible by UV transilluminator (Desselberger, 1995.).

In our experiments, we used Ni primer (21 bases, G + C = 57%) and E3 primer (17 bases, C =35% , without G). This may be the main reason for non-specific amplification . Using of other primer instead E3 primer and performing of PCR optimization regarding primer annealing temperatures, will probably reduce the appearance of non-specific PCR amplification products, in further experiments.

The combination of SRSVs-RT-PCR detection with broadly reactive primers Ni/E3 followed by genotyping (genogroup specific primers GI/GII/E3 and DNA sequencing is very efficacious approach in investigation of environmental contamination and the epidemiology of SRSVs infectiuons in the community (Gray et al., 1997.).

This study confirms high level of sensitivity in SRSVs-RT-PCR diagnosis in cases of acute gastroenteritis at infected humans.

Apstrakt

UPOTREBA REAKTIVNOG PRAJMERA RT-PCR U OTKRIVANJU MALIH OKRUGLIH VIRUSA

Mali okruglo-strukturirani virusi (Small round structured viruses ili SRSVs) su uzrocnici nebakterijskog akutnog gastroenteritisa kod ljudi. Clanovi su familije Caliciviridae, te su slicni po osnovu svoje velicine sa picornavirusima (28-30 nm) i specificni po okruglasto omedjenim podrucjima na povrsini partikule. Virion sadrzi jednostruku, pozitivno

orjentisanu RNK (7.4 – 7.7 kb) i nemaju lipoproteinsku ovojnicu. U stvari, SRSVs su clanovi NLVs roda (Norwalk-like virusi) familije Caliciviridae.
SRSVs sojevi virusa su: NV-8FIIa/68/US; KY-89/89/9; Sa-1283/84/9; SMA/76/US; 925/92/UK; OTH-25/89/9. Aktualni SRSVs oligonukleotidni detekcioni primeri su oznaceni brojevima na primjer 36, 35, 69, 78 itd. I za njih je tacno utvrdjena pozicija na 5′ kraju genomske RNK. Takodjer su dizajnirani primeri za koje jos nije utvrdjena prtecizna lokacija unutar SRSV genoma (na primjer primeri oznaceni brojevima 86, 95, 96 itd.).
Dobijeni rezultati, prezentirani u ovom radu, pokazali visoku senzitivnost i specificnost molekularne SRSVs detekcije u uzorcima fecesa klinickih pacijenata sa specificnim klinickim simptomima akutnog gastroenteritisa, pomocu siroko reaktivnih oligonukleotidnih primera (Ni i E3), prilikom koristenja RT-PCR metoda amplifikacije i detekcije PCR-SRSV produkata pomocu UV transiluminatora a nakon elektroforetske separacije u agaroznom gelu.
Kljucne rijeci: SRSVs, gastroenteritis, RT-PCR.

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