Journal of Clinical Virology 44 (2009) 211–214

Contents lists available at ScienceDirect

Journal of Clinical Virology

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

PCR inhibition in stool samples in relation to age of infants

Sami Oikarinen[a,b,*], Sisko Tauriainen[a,b], Hanna Viskari[a,b], Olli Simell[a,c], Mikael Knip[a,d,e], Suvi Virtanen[f,g,h], Heikki Hyöty[a,b,i]

[a] JDRF Center for the Prevention of Type 1 Diabetes in Finland, Finland
[b] Department of Virology, Medical School, University of Tampere, Biokatu 10, 33520 Tampere, Finland
[c] Department of Pediatrics, University of Turku, Turku, Finland
[d] Department of Pediatrics, Tampere University Hospital, Tampere, Finland
[e] Hospital for Children and Adolescents, University of Helsinki, Helsinki, Finland
[f] Tampere School of Public Health, University of Tampere, Finland
[g] Department of Health Promotion and Chronic Disease Prevention, National Public Health Institute, Helsinki, Finland
[h] Tampere University Hospital Research Unit, Tampere, Finland
[i] Department of Clinical Microbiology, Centre of Laboratory Medicine, Tampere University Hospital, Tampere, Finland

ARTICLE INFO

Article history:
Received 16 December 2008
Accepted 18 December 2008

Keywords:
PCR inhibition
Internal control
Stool sample
Age
Dietary
BSA

ABSTRACT

Background: PCR is rapidly replacing traditional methods in diagnostic virus laboratories. PCR inhibitors, which are often present in clinical samples, may lead to false negative test results.
Objectives: The aim was to study the presence of PCR inhibitors in stool samples collected from 3- to 24-month old children.
Study design: Total RNA fraction extracted from stool samples was spiked with a standardized amount of Semliki Forest Virus RNA and amplified using specific PCR primers. The presence of PCR inhibitors was detected by a decrease in amplification rate compared to spiked water samples. Inhibition in different age groups and dietary origin of PCR inhibitors were analyzed by comparing the samples taken during exclusive and non-exclusive breastfeeding periods. The inactivation of PCR inhibitors was also assessed.
Results: Complete inhibition was seen in 12% (13/108) and partial inhibition in 19% (21/108) of the samples. Inhibition was seen in none of the stool samples (0/31) taken from infants younger than 6 months compared to 17% of samples (13/77) taken from 6 to 24 months old infants ($p < 0.036$). Breastfeeding was more common in younger age group. Addition of bovine serum albumin (BSA) into the reaction mixtures eliminated the effect of inhibitors leading to all samples being positive.
Conclusions: PCR inhibitors are frequent in stool samples. They may originate from dietary components and can lead to false negative PCR results. The addition of BSA to the cDNA and PCR reactions proved to be an easy and effective method for eliminating the inhibitory effect of these compounds.

1. Background

Methods based on polymerase chain reaction (PCR) have replaced many traditional virus detection assays in clinical virus laboratories. In spite of their superior sensitivity, they have also weaknesses, which limit their use in virus diagnostics. One of these problems is caused by PCR inhibitors, which are often present in clinical samples and may lead to false negative results.[1] Such inhibition can be detected using an internal control in the PCR reaction.

Although PCR inhibitors seem to be common in stool samples, disturbing detection of viruses and other microbes, little attention has been paid on the potential biases caused by these poorly defined PCR inhibitors. The quality and quantity of inhibitors varies between samples, and several kinds of PCR inhibitors have been characterized including phenolic compounds, glycogen, fats, cellulose, constituents of bacterial cells, non-target nucleic acids and heavy metals.[2,3] However, it is not known if the concentration of these inhibitors in stool samples correlates with differences in dietary factors, gut microbiota or other factors in our environment or lifestyle. Furthermore, previous studies aiming at characterization of these inhibitors have been based on small sample series. Indeed, the overall analytical impact of PCR inhibitors is still a matter of controversy.

Abbreviations: PCR, polymerase chain reaction; RNA, ribonucleic acid; BSA, bovine serum albumin; cDNA, complementary DNA; DIPP, Finnish Type 1 Diabetes Prediction and Prevention study; HBSS, Hank's balanced salt solution; SFV, Semliki Forest Virus; BHK-21, Syrian hamster kidney cell; dNTP, deoxyribonucleotide triphosphate; DNA, deoxyribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; cps, counts per second.

* Corresponding author. Tel.: +358 3 2158459; fax: +358 3 2158450.
E-mail address: Sami.Oikarinen@uta.fi (S. Oikarinen).

1386-6532/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jcv.2008.12.017

Various methods have been developed to remove or inactivate these inhibitors. Many nucleic acid extraction methods can eliminate part of the PCR inhibitors but their efficiency varies and is far from complete. Consequently, the selection of the nucleic acid extraction method is a trade-off between sensitivity of nucleic acid extraction and the efficacy of removal of inhibitors.[4] Previous studies have shown that inhibitors can partly be inactivated or bound by several compounds such as betaine, bovine serum albumin (BSA), formamide, glycerol, gp32, nonidet P-40 and tween.[5] The amount of PCR inhibitors can also be reduced after nucleic acid extraction, but these methods are time consuming and reduce the yield of nucleic acids, limiting their value in clinical diagnostics.

2. Objectives

This study evaluates the frequency and effect of PCR inhibitors in a large number of stool samples to assess how frequently they could lead to false negative findings in PCR analyses. In addition, we analyzed inhibition in different age groups and how these inhibitors correlate with the diet (simple diet based on breastfeeding vs. complex diet based on supplementary food). Finally we studied the efficiency of BSA in the removal of these inhibitors.

3. Study design

3.1. Subjects

Stool samples ($N = 108$) were collected from 27 infants aged from 3 to 24 months (mean age 10 ± 6 months) during their prospective observation starting from birth. These children participated in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study.[6] For the first 2 years of the child's life the family was asked to record the age at introduction of all new foods and end of breastfeeding on a continuous form, which was checked by trained study nurses at 3, 6, 12, 18 and 24 months visits.[7] The children were categorized into two groups: children who were exclusively breastfed, and children who received supplementary food.

3.2. Nucleic acid extraction

Stool specimens were first suspended (1:10) into HBSS (Hank's balanced salt solution) supplemented with gentamycin sulphate (Biological Industries, Israel), Fungizone (Amphotericin B), penicillin G, and 4% BSA (Calbiochem, USA) and RNA was extracted from 140 μl of this suspension using silica columns (QIAamp Viral RNA Kit, Qiagen, Hilden, Germany). From selected samples RNA was extracted also by MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany) using automated nucleic acid extraction robot (MagNA Pure LC Instrument, Roche, Mannheim, Germany), which is based on magnetic coated glass particles. Extractions were performed according to the manufacturer's protocols.

3.3. Detection of PCR inhibition

The nucleic acid fraction which was extracted from the stool sample suspension was first spiked with a standardized amount of Semliki Forest Virus (SFV) RNA prior to RT-PCR reaction. This SFV RNA was originally extracted from SFV-infected Syrian hamster kidney (BHK-21) cells using silica columns (QIAamp Viral RNA Kit) and was added to the cDNA mastermix to ensure that it was present in equal titres in all experiments (100,000 copies of SFV RNA/reaction). SFV RNA diluted in water was used as a positive control, to which the magnitude of possible inhibition was compared (Fig. 1). The RT reactions were performed using RT-buffer

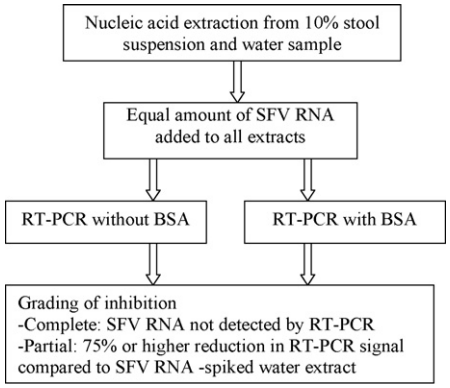

Fig. 1. Summary of study design.

(Promega, Madison, WI, USA), 0.5 mM dNTP (Pharmacia Biotech, Uppsala, Sweden), 4 U of RNase inhibitor (Promega), 50 pmol SFV-specific reverse primer (5′-TGCATGGTCATTTGGTGTGACC-3′), 20 U of Moloney murine leukemia virus reverse transcriptase enzyme (Promega) and 10 μl of the RNA template. Parallel reactions were performed with 0.5% of BSA added to the reaction mixture. The total volume of RT-PCR reaction was 40 μl. Reactions were incubated at 37 °C for 60 min. PCR reactions were done using 0.2 mM dNTP, 0.2 μM of SFV-specific primers (biotinylated forward primer 5′-ATGGCGGATGTGTGACATAC-3′; reversed primer 5′-TGCATGGTCATTTGGTGTGACC-3′), 1 U of DyNazyme DNA Polymerase, (Finnzymes, Espoo, Finland) and 10 μl of template cDNA. Parallel reactions were performed using 0.35% of BSA in the PCR mixture. The total reaction volume was 100 μl. cDNA template and primers were denaturated at 94 °C for 3 min, followed by 40 cycles each consisting at 94 °C for 30 s, at 53 °C for 45 s and at 72 °C for 1 min. Final extension was at 72 °C for 7 min. PCR amplicons were linearly quantified by a liquid-phase hybridization assay[8] using a samarium labelled probe (5′-GACGGAAATGCCTTCTG-3′) (PerkinElmer, Turku, Finland) specific for SFV.

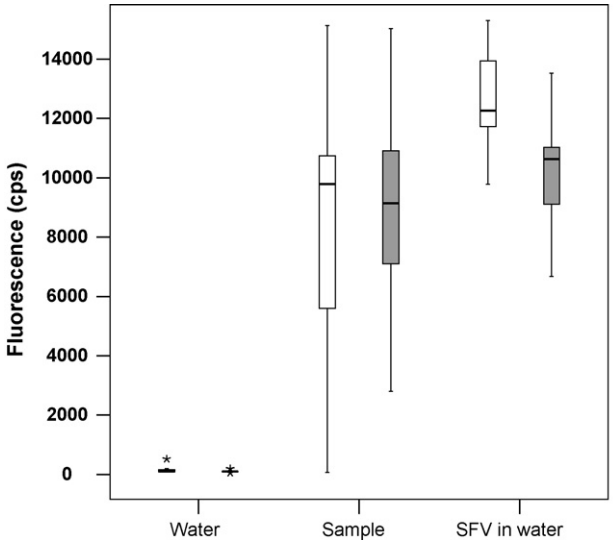

Fig. 2. Amplification of Semliki Forest Virus RNA from spiked stool and water samples using RT-PCR. The *Y* axis represents detection of amplification products using a Semliki Forest Virus specific probe in a liquid-phase hybridization assay (fluorescence counts per second). White bars indicate results obtained without BSA in RT-PCR reaction and gray bars results with BSA. Negative control represents background signal of non-spiked water control. The median for each dataset is indicated by the black center line, and the box presents the middle 50% of the dataset. The ends of the vertical lines indicate the minimum and maximum data values, unless potential outliers are present as asterisk.

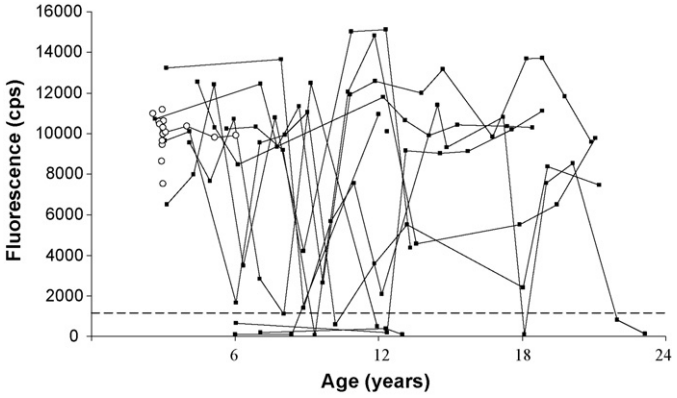

Fig. 3. Effect of age and diet on the level of PCR inhibitors in stool samples. White circles indicate exclusive and black circles non-exclusive breastfeeding samples. Sample series collected from each individual are connected with line. Dash line indicates the detection limits.

3.4. Statistical analyses

Frequency of inhibited samples was analyzed under 6 months and from 6- to 24-month old groups. Firstly, means of both groups were calculated for every child and further the means were tested using Wilcoxon test. A p value <0.05 was regarded significant.

4. Results

The presence of RT-PCR inhibitors was first tested in 108 stool samples. The amplification of SFV RNA was clearly lower in stool sample extracts compared to water sample extracts. Median fluorescence intensity in the hybridization assay was 12.268 counts per second (cps) in water samples and 9.790 cps in stool sample extracts (Fig. 2). When the cut-off limit for SFV RNA positive samples was set to a level representing five times the cps of non-spiked water sample extract (this is usually used as the cut-off limit in our diagnostic PCR tests), the amplification of SFV RNA (100,000 copies of SFV RNA per reaction) turned completely negative in 12% (13/108) of the stool samples. In addition, almost complete inhibition (75% or higher reduction in cps values) was detected in 7% (8/108) of the samples. Accordingly, altogether 19% (21/108) of the stool samples included such a high amount of PCR inhibitors that a false negative result in diagnostic RT-PCR assays was possible. The presence of PCR inhibitors in the stool samples was tightly associated with age of the child, as none of the 31 stool samples taken from younger than 6 months old infants were negative, whereas 13 (17%) out of the 77 samples taken from children aged 6–24 months were inhibited ($p < 0.036$). In the age group of less than 6 months, 61% of the samples were collected in the exclusive breastfeeding period, in the age group from 6 to 24 months only one sample was collected in the period of breastfeeding, the sample was not inhibited (Fig. 3).

All samples were also tested in parallel using BSA in the RT and PCR reactions. Addition of BSA eliminated the RT-PCR inhibition effectively leading to comparable results in SFV RNA-spiked stool and water samples (median 9.146 cps vs. 10.630 cps; Fig. 2). Even the 13 samples with complete inhibition turned clearly positive after the addition of BSA to the RT-PCR reactions (median 182 cps without BSA vs. 8.506 cps with BSA).

We further analyzed the ability of two different nucleic acids extraction methods, silica columns and magnetic coated glass particles, to remove PCR inhibitors using the 13 samples, which contained the highest amounts of PCR inhibitors (complete inhibition in original SFV RNA amplification) as well as the 15 samples which showed no inhibition. Both methods performed equally well, although some variation between individual samples was seen (Fig. 4). The addition of BSA abolished inhibition equally well with both extraction methods.

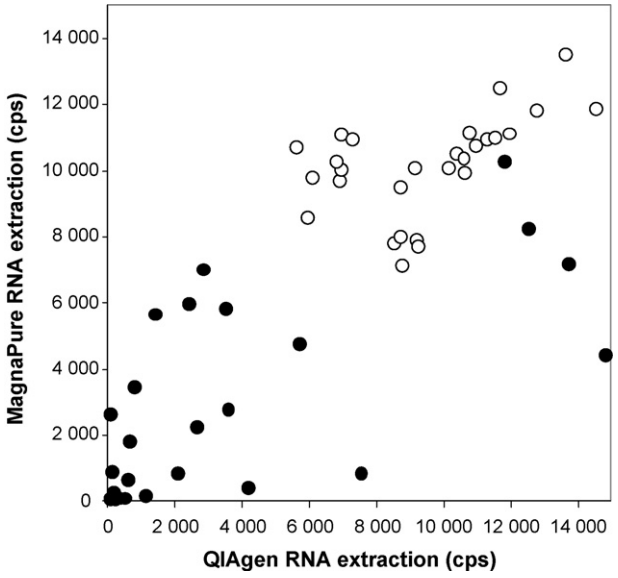

Fig. 4. Correlation between the two nucleic acid extraction methods in the detection of Semliki Forest Virus RNA in spiked stool sample without (black circle) and with (white circle) addition of BSA to RT-PCR reaction. The results of magnetic beads based method (MagnaPure RNA extraction) are shown on the *Y* axis and the results of the silica based method (QIAgen RNA extraction method) are presented on the *X* axis.

5. Discussion

The present study shows that PCR inhibitors are relatively common in stool samples and that their effect can strongly influence the results of PCR assays. Altogether 19% of stool samples contained so high amounts of PCR inhibitors that they may lead to false negative results when viruses are searched for using a PCR-based assay. This creates a real problem in clinical virus laboratories as stool samples are quite commonly used for the detection of viruses. The amount of SFV RNA used in our study represents average viral RNA concentrations observed in stool samples during acute virus infections. SFV RNA was spiked into extracted RNA but not to the original stool sample to avoid the effect of possible variation in the RNA extraction step on the content of SFV RNA in the PCR reaction.[9]

In previous reports, the proportion of stool samples containing PCR inhibitors has varied substantially, ranging from 0% to 44%.[10,11,12] In the present study significant inhibition was observed in 19% of the samples. This variation may be due to different sample materials, methodologies (sensitivity to different inhibitors varies between polymerase enzymes) and the definition of the inhibition itself. The inhibition has usually been categorized as a binary variable, even though it is clearly a continuous variable. Thus, the presence of inhibition has also varied depending on the cut-off limit used in various studies.

The frequency of PCR inhibitors may be even higher in the adult population compared to that observed in the young infants, as we observed no inhibition in younger that 6 months old infants. This kind of effect can cause bias to the results in research projects where viruses are analyzed from stool samples, especially if an age of the study participants varies. This kind of age dependent effect on PCR inhibition has not been described earlier and further studies are needed to identify possibly connection to dietary and maturation of digestion system of infants which may be responsible for this effect. The present study emphases the need of internal controls to detect PCR inhibitors when clinical samples are analyzed using RT-PCR. In addition, one can try to eliminate the effect of these

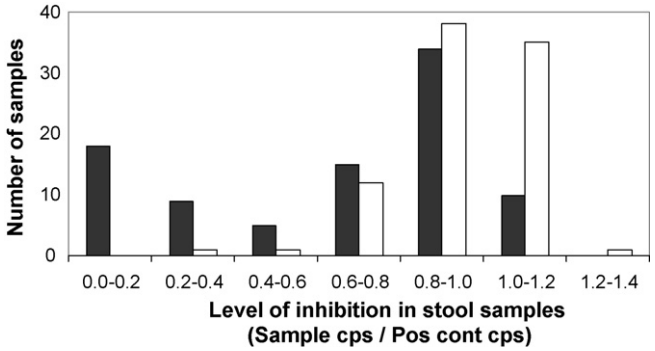

Fig. 5. Shift in PCR amplification after addition of BSA. Black bars indicate results obtained without BSA in RT-PCR reaction and white bars results with BSA.

inhibitors. We tested the effect of BSA added to both RT and PCR reactions as a factor eliminating the effect of inhibitors, as a previous study has suggested that BSA may have such a favourable effect.[13] BSA treatment decreased the efficacy of PCR amplification in samples, which did not contain any PCR inhibitors (water samples spiked with SFV RNA). However, all stool samples were positive when BSA was added to the RT and PCR reactions (Fig. 5). This indicates that the addition of BSA reduced the sensitivity of the RT-PCR method, but at the same time BSA effectively inactivated the RT-PCR inhibitors. Accordingly, the net effect of BSA was clearly favourable in stool samples eliminating the false negative samples. Based on these studies, we conclude that addition of BSA is an efficient method for decreasing false negative PCR results in clinical virus laboratories.

Conflict of interest

None declared.

Acknowledgements

We gratefully acknowledge Dr. Ari Hinkkanen's donation of the SFV strain and thank Heini Huhtala, M.Sc. for statistical analyses and Mrs Tanja Kuusela for expert technical assistance. We also thank the personnel of the DIPP project and highly appreciate the contribution of the families participating in the study.

Funding: The study was supported by Juvenile Diabetes Foundation International, Academy of Finland, Medical Research Fund of Tampere University Hospital, Tampere Graduate School in Biomedicine and Biotechnology and the City of Tampere. *Ethical approval*: Ethical approval was given by Ethical Committee of Pirkanmaa Hospital District Judgement's reference numbers: 97193M, 99078, 99077 and 99076.

References

1. Valentine-Thon E. Quality control in nucleic acid testing-where do we stand? *J Clin Virol* 2002;**25**(Suppl. 3):13–21.
2. Lofstrom C, Knutsson R, Axelsson CE, Radstrom P. Rapid and specific detection of Salmonella spp. in animal feed samples by PCR after culture enrichment. *Appl Environ Microbiol* 2004;**70**:69–75.
3. Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, et al. Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. *J Clin Microbiol* 1997;**35**:995–8.
4. Hale AD, Green J, Brown DW. Comparison of four RNA extraction methods for the detection of small round structured viruses in faecal specimens. *J Virol Methods* 1996;**57**:195–201.
5. Al-Soud WA, Radstrom P. Purification and characterization of PCR-inhibitory components in blood cells. *J Clin Microbiol* 2001;**39**:485–93.
6. Kupila A, Muona P, Simell T, Arvilommi P, Savolainen H, Hamalainen AM, et al. Feasibility of genetic and immunological prediction of type I diabetes in a population-based birth cohort. *Diabetologia* 2001;**44**:290–7.
7. Virtanen SM, Kenward MG, Erkkola M, Kautiainen S, Kronberg-Kippila C, Hakulinen T, et al. Age at introduction of new foods and advanced beta cell autoimmunity in young children with HLA-conferred susceptibility to type 1 diabetes. *Diabetologia* 2006;**49**:1512–21.
8. Lonnrot M, Sjoroos M, Salminen K, Maaronen M, Hyypia T, Hyoty H. Diagnosis of enterovirus and rhinovirus infections by RT-PCR and time-resolved fluorometry with lanthanide chelate labeled probes. *J Med Virol* 1999;**59**:378–84.
9. Verheyden B, Thielemans A, Rombaut B, Kronenberger P. RNA extraction for quantitative enterovirus RT-PCR: comparison of three methods. *J Pharm Biomed Anal* 2003;**33**:819–23.
10. Boom R, Sol C, Weel J, Lettinga K, Gerrits Y, van Breda A, et al. Detection and quantitation of human cytomegalovirus DNA in faeces. *J Virol Methods* 2000;**84**:1–14.
11. Beld M, Minnaar R, Weel J, Sol C, Damen M, van der Avoort H, et al. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. *J Clin Microbiol* 2004;**42**:3059–64.
12. Wilde J, Eiden J, Yolken R. Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions. *J Clin Microbiol* 1990;**28**:1300–7.
13. Al-Soud WA, Jonsson LJ, Radstrom P. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *J Clin Microbiol* 2000;**38**:345–50.