Establishment Real-time PCR for Detection Noroviruses in Stool Specimens

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Abstract Noroviruses are usually detected in stool specimens. Such samples commonly contain components reported to be (RT-) PCR inhibitors as well as the main inhibitory substance is the reverse transcriptase enzyme itself. This study provides a complex overview of the influence of RT systems, on the efficiency of PCR to detect Norovirus in stool specimens. The efficiency of three commonly used reverse transcriptases, namely Moloney Murine Leukemia Virus (M-MuLV), Avian Myeloblastoma Leukemia Virus (AMV) and Improm II reverse transcriptase (RT), have been compared to RT-PCR. The study used total RNA extracted from the same stool sample, no bias could therefore be attributed to sample preparation or host individuality. All amplification reactions were run using starting material from the stock, 1/10, 1/100 and 1/1000 cDNA dilutes.

In the results, Improm II yielded very weak bands, in the presence of the stock cDNA and completely failed to amplify DNA in the presence of the 1/10, 1/100, 1/1000 cDNA dilute. Whereas AMV yielded bands in the presence of the stock and 1/10 cDNA dilute and M-MuLV yielded bands in the presence of the stock and all cDNA dilutes.

Keywords Noroviruses; Reverse transcriptase; PCR; Stool

Background Noroviruses are a major cause of epidemic and sporadic gastroenteritis in humans and clinical signs and lesions of gastroenteritis were reported in bovines. The full-length sequencing of different human Norovirus genomes has allowed the development of reverse transcription polymerase chain reaction (RT-PCR) which has become the gold standard for Norovirus diagnosis in stool and environmental samples (Atmar and Estes, 2001; Jiang et al., 1993; Lambden et al., 1993). With such samples, the control for inhibition of the reaction during amplification and detection is crucial to avoid false negative results, which might otherwise not be detected (Scipioni et al., 2008).

For RNA detection, the most fundamental step is the in-vitro reverse transcription (RT) of RNA to its complementary DNA (cDNA), catalyzed by the enzyme reverse transcriptase (Arezi and Salehi, 2006; Kawasaki, 1990; Sellner et al., 1992; Verma, 1997). Many factors such as the quality of the RNA, the presence of inhibitor co-extracted with the RNA, as well as the RT efficiency are known to affect the total yield of cDNA (Bustin and Nolan, 2004; Arezi et al., 2010). Some studies have shown the main inhibitory substance is the reverse transcriptase enzyme itself (Chandler et al., 1998; Chumakov, 1994). As such, the enzyme needs to be removed or denatured directly after the RT reaction is complete to reduce its inhibitory and/or nuclease activities. This can be achieved via a heat or an alternative inactivation step, which has been incorporated in almost all commercially available reverse transcriptase kits/systems. However, with the increasing number of heat-resistant RT enzymes (Arezi et al., 2010), and the fact that many RT enzymes retain their inhibitory-resistant RT enzymes even post denaturation (Suslov and Steinleider, 2005), there is a need for alternative strategies to overcome this limitation. Dilutions of RT products (cDNA) are often used to minimize post-RT inhibitory effects (Sellner et al., 1992; Levesque et al., 2007; Chandler et al., 1998; Chumakov, 1994; Wilson, 1997), although this has the negative consequence of reducing quantification precision, which is especially problematic when dealing with very low copy templates (Okello et al., 2010).
Evaluating the capacity and detection limits of different commercial RT systems in regard to these conditions is fundamental to maximizing the sensitivity of the RT-PCR (Sergerie et al., 2007).

For that reason, for detection Norovirus in stool samples the first stage of gene expression analysis, the RT step, was examined in order to give further directives about the appropriate choice of the RT system for RT-PCR assay.

The aim of this work was to study the sensitivity of some commercially available reverse transcriptases which able to detect Noroviruses circulating in the human and bovine populations.

1 Results

1.1 Agarose gel electrophoresis

A PCR reaction was considered positive if a 319 bp band could be visualized on an agarose gel following electrophoresis; however, in many instances DNA amplification success (band intensity) was a continuum, decreasing as the inhibitor concentration increased.

1.2 AMV reverse transcriptases (RT)

For the RT-PCR amplification of cDNA, the AMV reverse transcriptases were able to produce bands from stock and 1/10 diluents (Figure 1).

1.3 Improm II reverse transcriptases (RT)

Improm II reverse transcriptases could only produce a trace amount of stock cDNA amplification products (Figure 2).

1.4 M-MuLV-Reverse Transcriptase (RT)

For the RT-PCR amplification of cDNA, M-MuLV reverse transcriptases were able to produce bands from stock and all cDNA dilutions (Figure 3). We demonstrated that the use of M-MuLV reverse transcriptases was superior to AMV and Improm II reverse transcriptase for the synthesis of Noro virus cDNA from stool samples. We show that reverse transcription yields vary up to 1000 fold with the M-MuLV reverse Transcriptase.

2 Discussion

Feces constitute complex biological samples, which cause problems when PCR is used as a diagnostic method, not only because of the presence of numerous types of bacteria but also because of the different kinds of food, degradation products present (Greenfield and White, 1993).

Messenger RNA profiling relies heavily on a reverse transcription step, which is accompanied by PCR. The recombinant Moloney murine leukemia virus M-MuLV, Improm II and avian myeloblastosis virus AMV-RT, are the most frequently used reverse transcriptases for this purpose. There is a well-known inhibitory effect of PCR by RT components (Sellner et al., 1992;
Fehlmann et al., 1993; Chumakov, 1994; Chandler et al., 1998; Liss, 2002). The introduction of micrograms of RNA into the RT step, followed by the extensive dilution of RT reaction before PCR execution, is supposed to minimize this inhibition (Sellner et al., 1992; Chumakov, 1994; Chandler et al., 1998; Aatsinki et al., 1994; Karsai et al., 2002). These conditions often are not met for two reasons: there is not enough RNA available, and the extensive dilution will negatively affect the precise detection of rarely expressed genes (Suslov and Dennis, 2005).

In this study, we addressed the efficiency of different commercial RTases and assessed their sensitivity in their respective systems, following the vendors’ recommendations. We found that, whereas the RNA-to-cDNA conversion of abundant transcripts is efficient with all commercial RT systems tested in the presence of various amounts of background RNA, cDNA could not be detected with Improm II RT systems when high cDNA diluents are involved. We found that the RT step is critical for gene analysis and that a judicious choice is required when starting RNA material is limited.

In this study, M-MuLV reverse transcriptases were able to produce bands from stock and all diluents cDNA. Loisy et al (2005) used M-MuLV reverse transcriptases for Norovirus detection after RNA extraction from Shellfish sample. Jean et al (2003) used M-MuLV reverse transcriptase for detection of Norovirus genogroup in stool samples. Kellogg et al (2001) used avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) for detection of Norovirus genogroup in stool and Shellfish samples. Pang et al (2004) used SuperScript II RNase H reverse transcriptase kit (Invitrogen) for detection of Norovirus in stool samples, SuperScript II Reverse Transcriptase (RT) is an engineered version of M-MuLV. Kageyama et al (2003) used SuperScript II RNase H reverse transcriptase (Gibco BRL, Gaithersburg, Md) for Norovirus detection after RNA extraction from stool samples. Dreier et al (2006) used SuperScript™ III Reverse Transcriptase (Invitrogen) for Norovirus detection after RNA extraction from stool samples. SuperScript™ III Reverse Transcriptase is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability. The enzyme is purified to near homogeneity from E. coil containing the modified pol gene of M-MuLV.

In this study, AMV was able to produce bands from stock and 1/10 cDNA. The addition of BSA, that is able to scavenge a variety of inhibitory substances (Kreader, 1996), (Wise et al., 2003) and Trujillo et al (2006) used Avian myeloblastosis virus (AMV) super reverse transcriptase (Molecular Genetic Resources, Tampa, FL) for Norovirus detection after RNA extraction from stool samples. Jiang et al (1999) used AMV-RT enzyme after RNA extraction from stool samples. Richards et al (2004) used DuraScript reverse transcriptase (enhanced avian myeloblastosis virus) for Genogroup I and II Noroviruses Detected in Stool Samples.

Christian Beuret (2003) used Sensiscript-RT kit for detection of Norovirus genogroup in stool samples. Hoehne and Schreier (2006) used Omniscript and Sensiscript reverse transcriptases for detection of Norovirus genogroup I and II in stool samples.

In conclusion, these enzymes differed significantly in their efficiency of cDNA synthesis as indicated by the degree of amplification by PCR; Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase produced greater amplification using RT-PCR than that of the Avian Myeloblastoma Leukemia Virus (AMV) and Improm II reverse transcriptase.

3 Materials and Methods

3.1 Stool samples and RNA extraction

The PCR assay was optimized using archived positive human stool sample, a stool suspension 10% in sterile phosphate-buffered saline (PBS) was performed, RNA was extracted from PBS suspension with the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the column centrifugation procedure described by the manufacturer. The amount of RNA in the extracted material was measured using a NanoDrop spectrophotometer.

3.2 DNase treated RNA

To reduce nonspecific amplification and to prevent carryover contamination by Norovirus cDNA, RNA was treated with DNase I before RT, for this viral RNA (12.5 µL) was added to the reaction mixture.
(2.5 µL) containing DNase I buffer and 1 U of RQ1 DNase (Promega). The reaction at 7 mixture was incubated at 37°C for 30 min to digest DNA and then 5°C for 5 min to inactivate the enzyme.

3.3 Reverse TRANSCRIPTION (RT)
To address the efficiency of commercial RT systems, we set up an RT-PCR system that could independently monitor the influence of reverse transcriptase. The commercial RT systems tested were Improm II, AMV and M-MuLV-Reverse Transcriptase (RT). The study used total RNA extracted from the same sample and method. No bias could therefore be attributed to sample preparation or individuality.

3.3.1 Improm II reverse transcription (RT)
The reverse transcription was performed in two steps. For the first step, 3 µL of RNA template was mixed with 6 µL nuclease-free water and 1 µL Random Hexamers primer and incubated at 70°C for 5 min followed by cooling to 4°C using a thermal cycler. For reverse transcriptase (RT) step, a total volume of 20 µL reaction mixture was prepared consisting of 10 µL RNA/primer mixture from the first step, 4 µL Improm II 5× Reaction Buffer, 2.4 µL 25 mM MgCl2, 1 µL dNTPs, 0.6 µL nuclease-free water, 1 µL RNease inhibitor, 1 µL Improm II reverse transcriptase. The mixture was returned to the thermal cycler and incubated at 25°C for 5 min, 42°C for 60 min and 70°C for 15 min before being cooled to 4°C.

3.3.2 AMV-reverse transcription (RT)
The reverse transcription reaction was performed in 50 µL of reaction mixture containing 5 µL of 10× PCR buffer (100 mM Tris–HCl pH 8.3; 20 mM MgCl2; 500 mM KCl; 0.01% gelatin), 5 µL of BSA (1%), 4 µL of dNTP mix (5 mM), 2 µL of negative sense primer (0.1 mg/mL), 0.25 µL RNasin (40 U/µL), 0.35 µL AMV-RT (20 U/µL), 31 µL of DEPC treated-water and 3 µL of the extracted RNA at 42°C for 1 hour.

3.3.3 M-MuLV-reverse transcriptase (RT)
The total amount of RT mix/sample was 47 µL, which contained the following components: 0.14 U/µL M-MuLV-Reverse Transcriptase, 0.2 U/µL RNasin, 3 ng/µL primer 289, 10 mM dNTPs; 0.5 mM MgCl2, 10×PCR Buffer [100 mM Tris-HCl (pH 8.3), 15 mM MgCl2, 500 mM KCl, 0.01% gelatin and RNase free water. 3 µL RNA was added to the RT mix, after which the mixture was incubated at 42°C for 1 hour.

3.4 cDNA suspension
All amplification reactions were set up using a series concentration of cDNA in nuclease-free water. Stock, 1/10, 1/100 and 1/1000 suspensions were carried out.

3.5 The primers
The primers used for the Real-Time PCR was from a published literature (Jiang et al., 1999); P 290 Forward: 5’–GATTACTCCAGTGAGCTCCCCAAC–3’ and P 289 Reverse: 5’–TGACAATGTAAATACATCACATATAC–3’.

3.6 Polimeras chain reaction (PCR)
For PCR a total volume of 50 µL the reaction mixture was prepared consisting of 25 µL Taq PCR MasterMix, 1 µL Revers primer (10 nM), 1 µL Forward primer (10 nM), 1 µL MgCl2 (25 mM), 5 µL cDNA and 17 µL nuclease-free water. The thermocycle program included 94°C for 3 min, 40 cycles at 94°C for 30 s, 49°C for 1 min 20 s and 72°C for 1 min, and a final 10 min extension at 72°C. The PCR product was analyzed by 1% agarose gel electrophoresis in 1× Tris-Asetat EDTA (TAE) Buffer with (1/10.000) ethidium bromide followed by visualization under UV light As a marker, a 100 bp ladder was used.

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Reference
Aatsinki J.T., Lakkakorpi J.T., Pietila E.M., and Rajaniemi H.J., 1994, A coupled one-step reverse transcription PCR procedure for generation of full-length open reading frames, Biotechniques, 16(2): 282-284, 286-288 PMid:7514006
Arezi B., and Salehi M., 2006, High quality external RNA control detects inhibitors in RNA samples, Strategies, 19: 52
Arezi B., McCarthy M., and Hogrefe H., 2010, Mutant of Moloney murine leukemia virus reverse transcriptase exhibits higher resistance to common reverse transcriptase-quantitative polymerase chain reaction inhibitors, Anal Biochem, 400(2): 301-303 http://dx.doi.org/10.1016/j.ab.2010.01.024 PMid:20100452
Atmar R.L., and Estes M.K., 2001, Diagnosis of noncultivatable, gastroenteritis viruses, the human caliciviruses, Clin Microbiol Rev., 14(1): 15-37 http://dx.doi.org/10.1128/CMR.14.1.15-37.2001 PMid:11148001 PMCid:88960
Bustin S., and Nolan T., 2004, Pitfalls of quantitative real-time reverse-
transcription polymerase chain reaction, Journal of Biomolecular Techniques, 15(3): 155-166 PMid:15331581 PMCid:2291693
Chandler D.P., Waggon C.A., and Bolton H. Jr., 1998, Reverse transcriptase inhibition of pcr at low concentrations of template and its implications for quantitative RT-PCR, Appl Environ Microbiol, 64(2): 669-677 PMid:9464406 PMCid:106100
Christian Beuret., 2004, Simultaneous detection of enteric viruses by multiplex real-time RT-PCR, Journal of Virological Methods, 115(1): 1-8 http://dx.doi.org/10.1016/j.jviromet.2003.09.005 PMid:14656455
Chumakov K.M., 1994, Reverse transcriptase can inhibit PCR and stimulate primer-dimer formation, Genome Res., 4: 62-64 http://dx.doi.org/10.1101/gr.4.1.62
Dreier J., Melanie S., Dietrich M., Sabine B., and Knut K., 2006, Enhanced reverse transcription-pcr assay for detection of norovirus genogroup I, Journal of clinical microbiology, 44(8): 2714-2720 http://dx.doi.org/10.1128/JCM.00443-06 PMid:16891482 PMCid:1594635
Fehlmann C., Krapf R., and Solioz M., 1993, Reverse transcriptase can block polymerase chain reaction, Clinical Chemistry, 39(2): 368-369 PMid:7679340
Greenfield L., and White T.J., 1993, Sample preparation methods, p. 122-137, In D.H. Persing, T.F. Smith, F.C. Tenover, and T.J. White (ed.), Diagnostic molecular microbiology: principles and applications, American Society for Microbiology, Washington, D.C.
Hoehne M., and Schreier E., 2006, Detection of Norovirus genogroup I and II by multiplex real-time RT-PCR using a 3'-minor groove binder-DNA probe, BMC Infectious Diseases, 6: 69 http://dx.doi.org/10.1186/1471-2334-6-69 PMid:16606447 PMcid:1524786
Jean J., Souza D., and Jaykus L., 2003, Transcriptional enhancement of RT-PCR for rapid and sensitive detection of Noroviruses, FEMS Microbiology Letters, 226(2): 339-345 http://dx.doi.org/10.1016/S0014-0106(03)00621-9
Jiang X., Huang P.W., Zhong W.M., Forkas T., Cubitt D.W., and Matson D.O., 1999, Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR, Journal of Virological Methods, 83(1-2): 145-154 http://dx.doi.org/10.1016/S0166-0934(99)00114-7
Jiang X., Wang M., Wang K., and Estes M.K., 1993, Sequence and genomic organization of Norwalk virus, Virol, 195(1): 51-61 http://dx.doi.org/10.1006/viro.1993.1345 PMid:8391187
Kageyama T., Kojima S., Shinohama M., Uchida K., Fukushi S., Hoshino F.B., Takeda N., Katayama K., 2003, Broadly reactive and highly sensitive assay for norwalk-like viruses based on real-time quantitative transcription-PCR, journal of clinical microbiology, 41(4): 1548-1557
Karsai A., Muller S., Platz S., and Hauser M.T., 2002, Evaluation of a homemade SYBR green I reaction mixture for real-time PCR quantification of gene expression, Biotechniques, 232: 790-792, 794-796
Kawasaki E., 1990, Amplification of RNA, In: Innis MA, Gelfand DH, Sninsky J.J., and White T.J., PCR protocols: A guide to methods and applications, San Diego, CA: Academic Press
Kellogg J.S., Frederick H.B., Françoise Le G., Mary K.E., and Robert L.A., 2001, Development of a reverse transcription-PCR-DNA enzyme immunoassay for detection of “norwalk-like” viruses and hepatitis a virus in stool and shellfish, Appl. Environ. Microbiol, 67(2): 742 http://dx.doi.org/10.1128/AEM.67.2.742-749.2001 PMid:11157239 PMCid:92643
Kreuder C., 1996, Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein, Appl Environ Microbiol, 62(3): 1102-1106 PMid:8975603 PMCid:167874
Lambden P.R., Caul E.O., Ashley C.R., and Clarke I.N., 1993, Sequence and genome organization of a human small round-structured (Norwalk-like) virus, Science, 259(5094): 516-519 http://dx.doi.org/10.1126/science.8380940 PMcid:8380940
Levesque-Sergerie J.P., Duquette M., Thibault C., Delbecchi L., and Bissonnette N., 2007, Detection limits of several commercial reverse transcriptase enzymes: impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR, BMC Molecular Biology, 8: 93 http://dx.doi.org/10.1186/1471-2199-8-93 PMid:17953766 PMCid:2151766
Liss B., 2002, Improved quantitative real-time RT-PCR for expression profiling of individual cells, Nucleic Acids Research, 30(17): e89 http://dx.doi.org/10.1093/nar/gfn088
Lissy F., Atmar R.L., Guillot P., Conn P.L., Pommepuy M., and Guyader F., 2005, Real-time RT-PCR for norovirus screening in shellfish, Journal of Virological Methods, 123(1): 1-7 http://dx.doi.org/10.1016/j.jviromet.2004.08.023 PMid:15582692
Okello J.B.A., Rodriguez L., Poinar D., Bos K., and Okwi A.L., 2010, Quantitative assessment of the sensitivity of various commercial reverse transcriptases based on armored HIV RNA, PLoS one, 5(11): e13931 http://dx.doi.org/10.1371/journal.pone.0013931 PMid:21085668 PMCid:2978101
Richards G.P., Michael A.W., Rebecca L.F., and Stephan S.M., 2004, Genogroup I and II noroviruses detected in stool samples by real-time reverse transcription-pcr using highly degenerate universal primers, applied and environmental microbiology, 70(12): 7179-7184
Scipioni A., Mauro A., Ziant D., Saegerman C., and Thiry E., 2008, A SYBR Green RT-PCR assay in single tube to detect human and bovine noroviruses and control for inhibition, Virology Journal, 5: 94 http://dx.doi.org/10.1186/1743-422X-5-94 PMid:18702817 PMCid:2546391
Sellner L.N., Coelen R.J., and Mackenzie J.S., 1992, Reverse transcriptase inhibits Taq polymerase activity, Nucleic Acids Research, 20(7): 1487-1490 http://dx.doi.org/10.1093/nar/20.7.1487 PMid:1374554 PMCid:312227
Sergerie J., Mathieu D., Catherine T., Louis D., and Nathalie B., 2007, Detection limits of several commercial reverse transcriptase enzymes: impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR, BMC Molecular Biology, 8: 93 http://dx.doi.org/10.1186/1471-2199-8-93 PMid:17953766 PMCid:2151766
Suslov O., and Steindler D.A., 2005, PCR inhibition by reverse transcriptase leads to an overestimation of amplification efficiency, Nucleic Acids Research, 33(20): e181 http://dx.doi.org/10.1093/nar/gni176 PMid:16314311 PMCid:1299832
Trujillo A.A., Karen A.M., Du-Ping Z., Leslie A.H., George V., Susan M.A., Tannie A., Roger I.G., and Stephan S.M., 2006, Use of tagman real-time reverse transcription-pcr for rapid detection, quantification, and typing of norovirus, journal of clinical microbiology, 44(4): 1405-1412
Verma I.M., 1977, The reverse transcriptase, BBA-Rev Cancer, 473: 1-38
Wilson I.G., 1997, Inhibition and facilitation of nucleic acid amplification, Appl Environ Microbiol, 63(10): 3741-3751 PMid:9327337 PMcid:168683
Wise G.A., Stephan S.M., Lora E.H., Danie L.G., Donald S., and Roger K.M., 2004, Molecular characterization of noroviruses detected in diarrheic stools of Michigan and Wisconsin dairy calves: circulation of two distinct subgroups, Virus Research, 100(2004): 165-177 http://dx.doi.org/10.1016/j.virusres.2003.11.014 PMid:15019235
Pang X.L., Bonita L., Linda C., Jutta K.P., and Stephan S.M., 2004, Evaluation and validation of real-time reverse transcription-PCR assay using the lightcycler system for detection and quantitation of norovirus, Journal of clinical microbiology, 42(10): 4679-4685 http://dx.doi.org/10.1128/JCM.42.10.4679-4685.2004 PMid:15472327 PMcid:522381

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