Format for ANSWERING REVIEWERS

Jul 31, 2015

Dear Editor,

Re: ESPS Manuscript NO: 20174

Title: Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis

Authors: Yupin Tong, Bonita E. Lee, Xiaoli L. Pang

We have reformatted the Title and Abstract according to the journal requirement. All the changes are highlighted with grey in the revised manuscript.

Below please find our responses to the reviewers' comments:

Response to reviewer 00504881

Manuscript Number	20174
Manuscript Title	Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis
Review Time	2015-06-15 23:28

Comments To Authors	The article describes a new assay for genotyping the human rotavirus. The results presented seem pretty convincing and conclusions are valid. Even though the clinical implications might be limited as the simultaneous assay for multiple genotypes cannot be performed by this assay (due to narrow range of Tm among the genotypes). Authors should describe the basic principle (mechanism) of using melting temp "Tm" as distinguishing feature for genotyping. Difference in "Tm" has been extensively used in bacterial and other microbial phylogenetics to identify a new species. In introduction- second paragraph- first sentence- VP14 should be VP1-4.
Authors' response	• The basic principle (mechanism) of using melting temp "Tm" as distinguishing feature for genotyping has been added in the manuscript as suggested by the reviewer (line 146-154) • "VP14" was corrected to "VP1-4". (line114)

Response to reviewer 00053556

Manuscript Number	20174
Manuscript Title	Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis
Review Time	2015-06-15 04:22

Comments To Authors	Comments to the Editor: Thanks for inviting me to review the article entitled "Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis". Comments to the authors: Minor Comment: o Minor editing polishing is needed o Language evaluation: "A". Comments to Authors: 1. TITLE Reflect the major content of the article 2. ABSTRACT It gives a clear delineation of the research background, including important data and conclusions; however, the aim of the study is not clearly identified. Also, the type of the samples was missing. 3. INTRODUCTION Provides sufficient background regarding the studied topic and the aim of the study is clearly identified 4. MATERIALS AND METHODS: Full description is provided for this section; however and in order to satisfy the reader, some important issues are better to be elaborated. o Subheadings for clinical specimens viral RNA extraction, R-T reaction and Real time PCR,.... were missing and are better to be maintained. o Preparation of stool suspension for RNA extraction is better to be included. o The detection limit and the detection range of the developed essay are better to be mentioned. o The source of positive controls of specific G and P genotypes has to be mentioned. o Statistical methods are missing and have to be mentioned. 5. RESULTS: ? An overall theoretical analysis of the study results is well covered. ? Provide sufficient experimental data, however, Paragraph 3 (lines 3-6): it is a discussion rather than results. This has to be revised ? Figures & tables are well presented 6. Discussion: The section is almost well organized; an overall theoretical analysis concerning the provided data is well covered, however, the results of the first paragraph were not clearly presented in the results section; specificity, sensitivity, correlation. 7-REFERENCES: Relevant and sufficient references were adequately cited and PMID/DOI is well maintained for all the cited references
Authors' response	• The abstract has been revised to the journal format stating the aim of the study. • Further details (subheadings, preparation of stool suspension, source of positive controls) have been added to the "Materials and Methods" as suggested by the reviewer. Since the objective of this study is to develop a SYBR Green real-time RT-PCR assay to genotype known positive rotavirus samples

	and not for primary detection of rotavirus, the limit of detection was not evaluated. We believe the data provided in comparing rotavirus genotyping using cnRT-PCR versus SYBR Green real-time RT-PCR was sufficient and appropriate for the study objective. • Results – subheadings have been added to provide more clarity in the result section including the data on specificity, sensitivity, correlation; part of the results have been moved to discussion section as suggested by the reviewer.

Response to reviewer 00504271

Manuscript Number	20174
Manuscript Title	Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis
Review Time	2015-06-10 12:02

Comments To Authors	The manuscript by Tong et al. introduced the new genotyping method of human Rotaviruses. This manuscript should be published because this method is worthwhile. This method can be applied for clade-analysis of closely related viruses, which the multiplex PCR method is not available as indicated in the manuscript (p. 8, l. 15-21). The figure legend is insufficient and should be revised properly.
Authors' response	• Figure Legend were revised

Response to reviewer 00503952

Manuscript Number	20174
Manuscript Title	Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis
Review Time	2015-06-10 04:57

Comments To Authors	The authors reported that ''The mean Tm (°C ± SD) for each of the genotypes were: G1 at 80.0°C±0.20, G2 at 80.9°C±0.49, G3 at 81.7°C±0.22, G4 at 80.7°C±0.20, G9 at 80.9°C±0.45, G12 at 80.6°C±0.37, P[4] at 80.7°C±0.50, and P[8] at 80.0°C±0.34.'' However, I noticed that G1=P[8]; and G2=G9; and from figure 1 and figure 2, I was not convinced that the mean Tm can be used to separate different genotypes of rotaviruses. Minor mistakes: On Page 4, ''which encodes six structure proteins (VP1-4, VP6 and VP7)'' should be ''which encodes six structure proteins (VP1-4, VP6 and VP7). There are some grammatical errors.
Authors' response	• As we have described in the manuscript, multiplex PCR could not be used for because the Tm of each genotype was too close to differentiate diversity of genotypes. Hence, we have chosen the approach of monoplex SYBR Green real-time RT-PCR for genotyping. The Tm is used to confirm if positive genotype was presented in each monoplex reaction. For example: If Tm is within 80.0°C±0.20 when using G1 primer set, It confirms the samples is G1, If Tm is within 80.0°C±0.34 when using P[8] primer set, It confirms the

	samples is P[8], etc. • "VP14" was corrected to "VP1-4". (line114)

Response to reviewer 00504174

Manuscript Title	20174 Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis
Review Time	2015-06-09 16:26

Comments To Authors	Tong and colleagues developed and validated a different approach to perform rotavirus G and P genotyping using a two-step SYBR Green real-time RT-PCR (rt-gPCR). In this effort the authors selected published cnRT-PCR genotype specific primers and optimized the amplification conditions using melting temperature to genotype the rotaviruses in the different samples. The sensitivity of the rt-gPCR, evaluated by using 16 samples of G and P genotypes, was the same of the conventional nested RT-PCR without cross-reactions with other gastroenteritis viruses. Using this techniques the authors genotyped 121 rotaviruses samples previously identified by elettron microscopy. In this analysis the authors reports different genotypes G1P[8] (42.6%), G2P[4] (4.9%), G3P[8] (10.7%), G9P[8] (10.7%), G9P[4] (6.6%), G12P[8] (23.0%), and unknown GP[8] (0.8%). Collectively this study remarks the importance of developing new sensitive and rapid molecular tecniques to monitor of rotavirus genotypes is important. However few points are not clear and need to be improved. Main point 1- In the first part of the results section, lines 9-11, the authors mentioned sequence data of the amplicons but they are not reported any methods used for sequencing. Why it is not reported the sequence data and specifities of the methods ? Are the sequences obtained previously ? 2- In the first part of the results section, lines 14-15, the authors should specify the type of the viruses. 3- In the first part of the results section, lines 18-19, the issue is not clear. 4- In the first part of the discussion, lines 6-14, what is the meaning of carry out a specific genotype examination

	before other genotype. This strategy has a longer turn-around-time. Why the authors have not considered to amplify shorter genomic regions using primers different from those published but able to genotype the different rotaviruses. Is it not possible because the regions are conserved ? the use of short regions (100-150 bp or lower) should improve the sensitivity and specificity of the assay. Minor points 1- Introduction, first page line 23. Conventional nested RT-PCR should be changed in "Conventional Reverse transcriptase nested PCR" 2- Introduction, second page line 9. Real time RT-PCR should be changed in "Reverse transcriprase (RT) Real time PCR" as in Materials and methods. 3- In matherials and methods, EM should be written completely. 4- All typos in the text should be corrected
Authors' response	• DNA sequencing methods and data analysis have been added in the revised manuscript. All PCR products generated by the new assay were sequenced (Sanger technique). The sequence data was uploaded in to RotaC tool (public free software) to define rotavirus genotypes. However, if sequences length is shorter than 500bp, the genotype cannot be determined using the RotaC tool. So for two G12 strains, we first blast in NCBI. We agree that with a BLASTn there could be an issue when errors are submitted to GenBank, but in our case, the first ten BLAST hits, were also G12 strains. In addition we picked one G12 hit in GenBank and analyzed it by the RotaC tool, and this confirmed the true G12 nature of this strain. • In first part of the results section, lines 14-15 - the types of virus used in the specificity test are clarified in the result section of the revised manuscript • In DISCUSSION second paragraph - Because of monoplex PCR, we propose the use of "Tier Testing Strategy" to improve the turnaround time and save the costs. We would first test for the most predominant genotype, e.g., G1 and P[8]. If positive results are generated, no further testing is needed. This strategy has worked very well in batch testing of samples. • Short amplicon design is mostly used in the qPCR probe to ensure specificity, which can be used to design multiplex PCR for simultaneous detection of a few targets in a single reaction tube. However, multiplex probe qPCR is not a good approach for genotyping rotavirus G and P types because there are too many different genotypes and the capacity of multiplex probe qPCR is limited. To design specific primers and probes for each genotype and optimize multiplex probe qPCR would be very challenging and involve high cost. The SYBR Green real-time RT-PCR

	validated in this study is superior to using shorter amplicons and multiplex probe PCR approach being more cost-effective. Moreover, PCR products would be available for down-stream tests e.g. DNA sequencing. • "RT" has been changed to "reverse transcriptase" and EM to "Electron Microscopy

Response to reviewer 00484099

Manuscript Title	20174 Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis
Review Time	2015-06-02 22:52

Comments To Authors	This article provides new an very useful tool for genotyping rotavirus. I recomend to show the results of sequencing, since they are demosntration that the technique works as it should
Authors' response	• This study is to focus on the new assay development so we don't think the sequence data is necessary. The whole VP7 or VP4 gene sequencing was not conducted in this study. The DNA sequences we obtained are based on the length of each genotype amplicon. We believe that sequence length of each genotype PCR product generated by the new SYBR Green real-time RT-PCR is sufficient to define correct genotype by using rota C tool and NCBI database.

Response to reviewer 70481

Manuscript Title	20174 Rapid genotyping of human rotavirus using SYBR Green real-time RT-PCR with melting curve analysis
Review Time	2015-06-02 16:00

Comments To Authors	The method described by the authors had been conducted by others to genotype other viruses or mRNA.But the work should be meaningful for the rotavirus genotyping.
Authors' response	• Thank you