Author's response to reviews

Title: The Limitations of Real-Time PCR Amplification for the Detection of Invasive Salmonella serovars in Blood and Bone Marrow Specimens from Enteric fever Patients

Authors:

Tran VT Nga (ngatvt@oucru.org)
Abhilasha Karkey (abhilashakarkey@hotmail.com)
Sabina Dongol (dongolsabina@yahoo.com)
Hang N Thuy (hangnt@oucru.org)
Sarah Dunstan (sdunstan@oucru.org)
Kathryn Holt (kh2@sanger.ac.uk)
Le TP Tu (tultp@oucru.org)
James I Campbell (jcampbell@oucru.org)
Tran T Chau (chautt@oucru.org)
Nguyen VV Chau (chaunvv@oucru.org)
Amit Aryjal (amitarjyal@yahoo.com)
Buddha Basnyat (rishibas@wlink.com.np)
Christiane Dolecek (cdolecek@oucru.org)
Jeremy Farrar (jfarrar@oucru.org)
Stephen Baker (sbaker@oucru.org)

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Author’s response to reviews:

Oxford University Clinical Research Unit
Hospital for Tropical Diseases
190 Ben Ham Tu, Quan 5
Ho Chi Minh City
Viet Nam
sbaker@oucru.org
Tel: (+84-8) 9 241 761
Fax: (+84-8) 9 238 904
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Dear Editor:

Enclosed is the modified manuscript entitled “The Limitations of Real-Time PCR Amplification for the Detection of Invasive Salmonella serovars in Blood and Bone Marrow Specimens from Enteric fever Patients” by Nga et al. The original manuscript was entitled “Comparison of Microbiological Culture and Real-time PCR for the Detection of Invasive Salmonella serovars in Blood and Bone
Marrow Specimens from Enteric fever Patients” and has been edited as recommended by the reviewers and we wish this modified article to be considered for publication in your journal.

The reviewer’s comments addressing our manuscript were both informative and generally well directed. Two of the four reviewers had some issues with the manuscript which we hope to clarify. However, this article has potentially important implications for the development of a good and reliable typhoid diagnostic. There are, as always with this type of study, some limitations, we attempted to take as many precautions to avoid any obvious limitations, but working on such samples is challenging and a constant issue for those working in laboratories in developing countries.

We have known for a while that PCR has significant limitations as a diagnostic assay for typhoid, and wished to highlight this as it is believed to be a highly sensitive and specific test. Previous publications for detecting DNA from invasive Salmonella in blood have substantial limitations, yet all conclude the assay is reliable. In truth, none of the previous tests would stand up to the rigors required in molecular diagnostic laboratories in industrialized locations. Nested PCR is unreliable due to issues with unspecific and simple contamination based matters, real time PCR is a robust amplification method permitting detection of a single copy of target DNA.

One of the issues associated with our findings is that it may seem simply that we cannot perform a simple amplification test or that our methodology is flawed. As readers will see from the data, it is robust and what we further show is that enteric fever is induced by an extraordinarily low amount of organisms circulating in the blood. Therefore, it is hugely challenging to try and amplify DNA from organisms which are either in low numbers or statistically not present in the sample prepared for amplification. Our findings may be judged to be somewhat controversial, owing to previously published work, but our study is scientifically sound and has important data. Publication of this article, we believe, is warranted for those using such tests and highlights obvious limitations in this methodology.

Each of the reviewer’s comments and questions are addressed in turn below.

Reviewer 1

Are the methods appropriate and well described? There are methods to test a test. Please refer to these methods to clarify the procedures performed. You have to use at least three groups of patients to test the test for clinical use: 1. True positive Typhi and Paratyphi patients: you use this group 2. False positive enteric fever by Typhi and Paratyphi patients: you could use other salmonella enteric fever patients. 3. True negative Typhi and Paratyphi patients: you could use a group of patients with other cause of fever: leptospirosis, brucellosis, malaria, etc.

This has been performed; the methodology has now been tested on a negative enteric fever group. This is included in the results section. Obtaining samples
from patients with other invasive Salmonella serovars was not possible in the setting used.

PCR test: 1. The primers designed are not located in any place of the Salmonella chromosome. Please give the target gene that you are looking for. Also, there are polymorphisms found in the genes used for Salmonella detection that can help to identify specific serovars. This work lacks of information of Salmonella genes used as targets and the characteristics that the authors used to design a specific primers and probes.

The probes and primers were designed to be highly specific for S. Typhi and S. Paratyphi respectively; both are on the respective chromosomes. The target genes are referred to in the text (by name) and the sequences are freely available from genome sequencing data. The probes and primers were designed within these genes and are presented in the text. I am unsure what further characterization is required by the reviewer.

The Ct of negative internal control is the same for positive internal control, it is confusing.

I am not entirely sure the reviewer understands the development of the assay, but agree that this may be a little confusing. A negative control cannot have a ct value (unless of course it is positive) we have modified table 2a to clarify that there was not interference of the internal control in the assay (with/without replaces + or – control).

The positive internal control is a very complicated plasmid obtained by recombination. This plasmid could be difficult to have available by other laboratories that can repeat the PCR. You can use a specific gene for Salmonella genus as internal positive control.

The internal control is a very simple plasmid with cloned gene into an E.coli strain. The idea behind this (a common and vital feature of a diagnostic PCR test) is that the organism is added prior to DNA extraction to ensure that extraction and amplification are both controlled during detection. We cloned a very unusual viral gene as we wished not to have any cross DNA homology with our target. i.e. we would not expect patients to have that organism in their blood. Once published, this plasmid and strain will be available to any groups requiring this as a reliable internal control. Using a Salmonella gene as an internal positive control would not be a positive internal control that would just be another detection marker. Meaning, if the PCR results were negative for the targets sequences, yet positive for your Salmonella internal control, your sample would have Salmonella DNA in the sample. This would be more confusing, the method we use means that if we do not get a positive control signal; either DNA extraction or amplification did not work. This is the most robust way of internally controlling the assay, rather than spiking with DNA.
Are the data sound? The authors have a very good number of positive patients, however the methodology used for test a test is not clear. Results are reporting in confusing way.

We have edited the results and the methods to improve clarity

Does the manuscript adhere to the relevant standards for reporting and data deposition? No

Are the discussion and conclusions well balanced and adequately supported by the data? No.

Are limitations of the work clearly stated? As the methodology used was not appropriate for the objective proposed, this work has big limitations.

I am unsure why the reviewer believes this; we have taken as many precautions as possible to ensure the work was performed in a controlled and robust manner. Our conclusion is, that in our setting the test is unreliable, and furthermore we show how many organisms are required for detection by growth and by PCR. The reviewer is directed to the papers we cite by Wain et al. and see that most patients have bacterial loads around 0.1 cfu/ml, therefore 10 mls of blood would have to be extracted and amplified for a positive PCR result.

Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? The authors cited several works about the specific topic, however the authors need to review the specific genes of Salmonella with the aim to develop a well funded PCR test for clinical diagnosis. Also there are many publications about detection of specific Salmonella serovars that are not included.

The genes we selected were selected by data mining the latest and most complete sequence data that was available. To ensure there was no cross-reactivity (which may be the case with previously published work). This work is as robust as we could make it and is more advanced than previous publications. We have cited all the main papers in the development of PCR tests for enteric fever.

Do the title and abstract accurately convey what has been found? No

These have been edited accordingly.

Reviewer 2

This manuscript describes a real time multiplex PCR assay to detect Salmonella serovar Typhi and serovar Paratyphi A from blood and bone marrow samples. The real time multiplex PCR assay target 131 bp segment of STY0201 and 104 bp segment of SSPA2308 for typhi and paratyphi serovars respectively. The authors have done substantial amount of work to validate the PCR assay by
spiking blood samples with different bacterial dilutions

Major compulsory revision. The glaring conclusion of the present work is the low sensitivity (42% for Typhi and 39% for paratyphi A). Quite a number of reports describing highly sensitive PCR assays are available. To cite a few, Sanchez-Jimenez et al, J. Med Microbiol (2004), 53, 875–878 have reported PCR assays which were more sensitive than the culture method. Similarly, PCR assays developed by Levvy et al J. Clin Microbiol 2008: 46, 1861-1866 also have 100% sensitivity and specificity. The authors further mention in the discussion section that PCR assay may be an alternative in situations where blood culture is not routinely performed. But with this low level of sensitivity, the present PCR assay cannot be an alternative for blood culture.

The point we wished to make is that PCR is being used as a diagnostic test in other laboratories as the reviewer describes. What we hoped to prove is that is not a limitation of our assay but a limitation of the technique as a diagnostic test. I realize that other laboratories have published high levels of specificity and sensitivity, but, we found the method to be unreliable and we wish to inform other groups of the precautions of performing unvalidated tests. I also agree with the last point, but some groups believe this test to be an appropriate substitute for blood culture, a working PCR would be useful but it is not an alternative for blood culture. This is what we are showing with the data presented.

The real time PCR assay has been evaluated in situations where the clinical enteric fever in patients is due to salmonella serovars of Typhi and Paratyphi A. Salmonella serovars other than these two serovars involved in systemic infection need to be tested; at least at organism level these be included to work out specificity. Otherwise the assay would not be even suggestive that enteric fever could be due to some other Salmonella serovar. The investigators probably need to reasses the real time PCR on to more appropriate clinical samples that should include the day of pyrexia samples before the initiation of rational antibiotic therapy. The spectrum of bacterial strains used for specificity testing should be increased by incorporating more strains of serovars like Paratyphi B, C, choleraesuis etc.

The primers and probes did not generate amplification from any common organism which can cause bacteriaemia (staph, strep, other salmonella), this is described in text, or indeed any other Salmonella serovars tested. Paratyphi B, C or Cholerasuis infection is very rare in the settings in which the study was based, therefore, these were deemed not only inappropriate, but we were unable to obtain a collection of sample from such patients. In the methods it now states that blood was taken before the use of antimicrobials. Also the later data
demonstrates that antimicrobials make little difference and all the patients that were culture positive, were culture positive for Typhi or Paratyphi A, therefore, the blood had organisms in on isolation.

In all the PCR assays the E. coli strain VU1 has been spiked into the blood inoculations and other bacterial dilutions for the purpose of internal amplification control (IAC). The normal practice is to incorporate a synthetic IAC construct in the PCR assays rather than spiking the E. coli strain harbouring the IAC construct. Real time PCR assays standardised with suitable concentration of primers and probe for the IAC also would improve the robustness of the assay.

Actually normal procedure and a more robust method is to spike the organism before extraction, thus controlling DNA extraction and amplification. How does the reviewer know if they have contaminated the sample or even if the DNA extraction has even worked? The concentration of the primers and probes was also evaluated and is shown in the methods.

The gene sequence targeted in the PCR assays should be sequenced and submitted in the data base.

The genes have been sequenced in a number of S. Typhi strains and S. Paratyphi A strains, hence the selection of the genes. These gene and sequences has been referenced and the genbank numbers added in the text, further addition to genbank is probably unnecessary, however we have the sequences available if required by the editor.

The geneeral conclusion derived by the investigators suggesting that the reported PCR do not have a high level of sensitivity when compared to culture cannot be accepted with the data presented. Even in our group we always find nearly 100% sensitivity of the reported PCR's with culture and antigen detection. The utility of this real time PCR assay can be improved by incorporating in the multiplex PCR assay a suitable gene target which is generic for salmonella serovars responsible for the enteric fever. The sensitivity could be improved by standardizing suitable DNA purification methods and improved sampling methods.

The DNA extraction methods are akin to previously published papers and the only way sensitivity could be improved is by DNA extraction from a larger blood volume. Actually, the results we find have been seen in other laboratories, one cannot amplify something that is not in the sample.

Minor essential revision

1. Materials and methods section: 1st para, The 7th line to be rephrased so that it does not begin with numeral.
Reviewer 3
This is a well written and important study examining the utility of PCR based methods for the detection of S. typhi and S. paratyphi in clinical specimens of blood and bone marrow. The methodology and controls are all appropriate and the data appears sound. The overall result is that even PCR has a limit to its detection rate. This study also calls into question other studies that propose that PCR can be use to detect S. Typhi in a clinical setting. One comment I have is on the specificity of the Salmonella internal control, what region or gene was this internal control designed from. And how many Salmonella serovars was it tested against?

This has been clarified in the text of the article. The internal PCR was from a phocid virus and was used as a marker for extraction and amplification. It was selected as this should not amplify Salmonella DNA from any serotype. The internal control was amplified from all the Salmonella serotypes along with the specific target sequences from Typhi and Paratyphi A.

One suggestion is that the title of the manuscript should reflect more the outcome of the study.

This has been changed to reflect the study findings.

Reviewer 4
A very well written paper. The objectives and rationale of the work were explained. Experiments were well-planned and executed. The methods were described clearly. To overcome the shortcoming of conventional, culture method, molecular approach such as PCR is now gaining importance and relevance in clinical diagnostic microbiology labs. The authors evaluated the primers and probes for sensitivity, specificity on DNA extracted from spiked specimens as well as from actual specimens. In spite of the ability of PCR to amplify any target, the authors noted that PCR on peripheral blood for Typhi many not be suitable due to the lack of sensitivity.
Additional comments.
As mentioned by the authors, typhoid fevers occur in areas of poor resourced areas. Real time PCR is much more costly (initial cost of acquiring a real time PCR machine as compared to a conventional PCR machine; more costly consumables) than the simple conventional PCR. Moreover, depending on the model or brand, some systems are closed system and that makes it more difficult for others to adopt similar strategy or approach unless one uses the same system. A conventional PCR would be more cost-effective and applicable in poor resourced laboratories. Once standardized and validated, a conventional multiplex PCR for simultaneous detection and differentiation of S Typhi, S.Paratphi, Salmonella (inclusive of an internal amplification control) would indeed be useful.

I agree with this comment and in our setting we have access to such machines. However, realtime PCR was established as it is known to be highly sensitive and may be adapted into a none quantitative method. This has been addressed in the discussion.

This version manuscript has been read and approved by all co-authors in its current form. We think you will find that the concepts of this article are novel, entirely consistent scope of your Journal and will be of significant interest to your readership. We hope you will consider publishing this article.

Thank you for your attention to this matter.

Yours truly,

Dr Stephen Baker