Author's response to reviews

Title: A Quantitative PCR (TaqMan) Assay for Pathogenic Leptospira spp.

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Version: 3 Date: 5 Jun 2002

PDF covering letter
RESPONSE TO REVIEWER'S REPORT

DR P LEVETT

A Quantitative PCR (TaqMan) Assay for Pathogenic Leptospira spp.
Lee Smythe
Version 2 – 10 May 2002

SPECIFIC QUESTION REQUIRING A RESPONSE

1. Materials and Methods Detection of PCR inhibitors, page 8; If I understand this section correctly, blood from a donor was collected into the tubes with various anti-coagulants and other treatments. Another interpretation is that serum was added in the laboratory to these tubes. A more precise description is required.

1. Comment
A more precise description has been provided and added as a correction to the manuscript to clarify whole blood from the donor was collected directly into the individual collection tubes. Serum was not added to these tubes in the laboratory.

2. If serum was used, the authors should consider repeating the experiment using blood, as hemoglobin may also inhibit the PCR reaction. In addition, most of the additives yield plasma, and it would be useful to demonstrate that the assay works when plasma is the specimen.

2. Comment
See comments (1) above.

3. The leptospiral DNA was spiked into the PCR mix, to which was added the DNA extracted from the blood. It would be more realistic to add leptospiral cells to the blood tubes before the extraction of DNA. Precise quantitative measurements would be more difficult to achieve in this way, but if necessary leptospiral DNA could be added in place of cells. What was the interval between addition of the blood and extraction of DNA from the samples?

3. Comment
We used this approach to ensure that there were standardised amounts of DNA which would correspond to known Ct values. Any differences between Ct values measured in the presence and absence of blood and the collection tubes was thus clearly due to inhibition of the PCR, and not due to variability in the lysis/ extraction of DNA from leptospiral cells. The interval between collection of the blood into the collection tubes and extraction was 4-5 hours.
4. **Results, Sensitivity and specificity, page 9:** The range of other bacterial species tested is restricted; 11 of the strains tested are from the Enterobacteriaceae and with the exception of one spirochete all are Gram negative bacilli. Similarly, a single non-pathogenic Leptospira serovar was tested. Moreover, strains from nine species of Leptospira were tested, but several species were not tested, including some which include both pathogenic and non-pathogenic serovars.

4. **Comment**
The range of bacterial has been expanded to include more Gram positive bacteria and non-pathogenic Leptospira. See corrected Tables 1 and 2. The bacterial species represented also reflect those agents more likely to be encountered in the infectious diseases and public health environments.

5. **This is not enough to support the conclusion that the assay discriminates between pathogenic and non-pathogenic strains. The representation of commonly recovered pathogenic serovars is good, however.**

5. **Comment**
The leptospiral strains have been expanded to include more representatives of the non pathogenic Leptospira to provide the discriminatory evidence required by the reviewer. See Table 2. We have modified the manuscript to make it clearer that our conclusion regarding the discrimination of non-pathogenic and pathogenic strains is tentative only.

6. **Discussion, page 11:** Clarify the sentence in paragraph 2 regarding differentiation between pathogenic and non-pathogenic leptospires; surely isolation and serologic identification is the way this has to be done? Provide a reference to support the statement that L.biflexa outgrows pathogenic species.

6. **Comment**
Our intention was not to minimise the importance of isolation and identification. Rather, we allude to the potential usefulness of culture-independent molecular methods as a complement to more traditional techniques. We have modified the manuscript to better illustrate our point. The statement that L. biflexa outgrows pathogenic species is both a personal observation gained from many years experience in a reference laboratory culturing the organism but more creditably supported by (Faine et al.,(1999) Leptospira and Leptospirosis, MediSci Publishing) where the doubling rate for a L.biflexa was described as 4.5 hours versus 6-8 hours for pathogenic leptospires. Though references are few, the another reference would be a 1947 publication by Shih Lu Chang “Studies on Leptospira Icterohaemorrhagiae” in the Journal of Infectious Diseases which makes note of the faster rate of growth of L.biflexa. **The author has withdrawn the statement.**
7. In a more general sense, why was 16S gene selected as the target for a diagnostic PCR? The ubiquitous distribution of this target is better suited to a broad-range assay in which specificity is not an issue. Perhaps the authors would consider this in the discussion.

7. Comment
The published sequence data was more abundant for the 16S gene which better facilitated the construction of a more specific probe and primer set for the PCR.
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ADVICE ON PUBLICATION

Unable to decide on acceptance or rejection until the authors have responded to the compulsory revisions.

This paper describes the evaluation of a Taqman PCR assay for the detection of pathogenic Leptospira spp. The application of this assay may be useful in early detection of infection before a serological response is produced, however there have been many published PCR assays for the detection of Leptospira spp.

Advice on publication: Comment
There have been published PCR assays for the detection of leptospires. However, as we pointed out in the Introduction, individual papers have reported PCR primers whose scope of detection is limited. Moreover, in the case of Woo et al—which is the only quantitative real-time study detecting leptospires—the work used only non-clinical samples. Indeed, the other reviewer of our manuscript states “This paper represents a step forward in leptospiral diagnostics. A real time molecular assay detecting pathogenic leptospires only is overdue and the authors are to be congratulated for their work.”

DISCRETIONARY POINTS

1. The sensitivity of the assay was assessed by adjusting the concentration of a cultured leptospiral cells using a counting chamber. These estimates appear optimistic and considering the inherent errors in estimating numbers can the difference between the detection limit of serum and urine be considered significant? The authors do not state the volume of eluate used to extract the samples using the Qiagen kit however, if it is assumed the extracted sample is recovered in 50ll then from an original sample volume of 200ll, the addition of 5ll of this extract represents 1/10 of the original 200ll sample (200ll). A minimum of 1 genome copy is obviously required for the PCR reaction to proceed. An absolute theoretical limit of 10 cells per 200ll or 500 per ml is therefore required and it seems unlikely the Qiagen column would reliably recover 1 or 2 cells. It is acknowledged that the ribosomal gene sequence is present in multiple copies which assists in detection, however the authors may like to review the stated lower limits of detection.

1. Comment
The use of the Petroff–Hauser counting chamber is the recognised gold standard (Faine et al., 1999, Leptospira and Leptospirosis, Second Edition, MediSci Publication) for the counting of leptospires. It is used regularly in our reference
laboratory to ensure cultures are used at optimum counts for accuracy of serological testing and classification of isolates. The volume of sample used in the Qiagen extraction was 200µl with a final recovered DNA sample volume of 200µl. A 5µl volume of this extract is used in the PCR reaction. The equivalent of two organisms in this 5µL volume was determined as the level of detection. This would be equivalent to the recovery of 80 organisms using the Qiagen kit and therefore would not be unrealistic as suggested. We understand that a reader could misinterpret our findings and have changed the manuscript (see Results paragraph 2 last sentence) to make it clear that the detection limit is stated for the PCR reaction not the initial sample.

COMPULSORY REVISIONS

2. The authors state they employ real-time (quantitative) PCR using Taqman chemistry to detect leptospires in clinical and environmental samples, however no data is presented to support this statement. This should be removed to suggest that it may be useful for detection from such samples but will require evaluation.

2. Comment

We have amended the discussion to include the word “may” indicating the potential usefulness of our method to detect leptospires in environmental samples. We have recently tested over 60 soil and water samples from field sites, however we feel that there is insufficient data to publish and hence have not referred to these findings in the manuscript.

3. Evidence to support the value of this assay in detection of leptospires from clinical samples was unconvincing. Presentation of the results should be made clearer with more relevant information. It is not stated what class of antibody the ELISA is detecting. If IgM is detected this may explain the difference in results with the MAT. More detail regarding the sera tested should be included. In particular, of the 66 samples tested how many were acute and how many were convalescent samples? It seems disappointing that not more of the samples were positive by PCR and of the 4 that were positive all were also culture positive and very high Ct values were recorded indicating very low levels of DNA.

3. Comment

The MAT detects all classes of antibody (Faine et al., 1999), whereas the ELISA referred to in this study detects only one class of antibody—in this case IgM. The positive anti-IgM ELISA results for this disease need to be validated by the MAT due to recognised problems of false positives with this type of assay (Faine et al., 1999). This is common knowledge and this difference may lead to significant differences in results. To maintain the logical thread of the argument of the manuscript, we saw little point in digressing to a discussion about the relative merits of these assays. Rather, we preferred to examine the merits of PCR for the diagnosis of leptospirosis correlated against the serology.

The evaluation and development of the PCR was based on the supply of clinically relevant specimens from a part of the country known to report the highest numbers
of human leptospirosis cases (Smythe et al., Commun.Dis.Intell., 24 (6):153-157) Specimens submitted for culture and PCR were assessed by the clinician as presenting in the bacteraemic phase of the disease offering the optimum chance of recovery of the organism. There is a very limited window of opportunity for isolation of the organism in this phase for humans (Faine et al., 1999). The integrity of the study required the collection of duplicate specimens of blood culture and sera from these patients at presentation. This is not the normal specimen collection practice and involved considerable rescheduling of collection orders for nursing and clinical staff as well as for laboratory staff storing, recording, handling and despatching specimens. As this aspect was conducted in a “real life” situation of patients presenting as patients, the acute and convalescent nature of the sera were unfortunately not readily available or unknown. The reference laboratory is one of the few laboratories in the world (sole laboratory in Australia) providing routine leptospiral isolation services. Numbers of isolates recovered annually are generally quite low and further hampered by the complexities of using specialised culture media and the need for whole blood collections for inoculation. The opportunity to confirm the PCR results by culture is therefore fortunate in this circumstance and the low numbers unavoidable.

4. **Considering whole blood was used for culture and only a small volume (2-5 drops) was able to recover viable leptospires, the authors might like to contemplate in the manuscript the possibility of increasing sensitivity by extracting DNA for PCR from whole blood samples.**

4. **Comment**
This is a good suggestion and something we would like to have done but the issues described in Point 3 and the complications introduced by storage, transport distance from the specimen source (over 3,000 kilometres) and need for regular deliveries made the use of viable whole blood samples difficult to achieve both physically and financially.

5. **The authors claim this assay can be used for detection of leptospires from urine, although this has not been demonstrated from clinical samples and this should be made clear.**

5. **Comment**
We agree and have changed the manuscript accordingly.

6. **Overall the evidence to support the view that this assay is useful for detection of leptospires from clinical samples is not convincing. It should be stated that much more work in evaluating clinical material needs to be undertaken.**

6. **Comment**
The PCR showed excellent correlation with isolation data and did not show false positives where antibody levels were present as indicated by a positive MAT. The evidence is equal or better to any previous published papers for PCR detection of leptospires from clinical samples. In most cases the patient numbers or sample numbers tested in this evaluation exceed or equate to publications on detection of leptospiral DNA by PCR. (Merien et al., (1992) J.Clin.Micro 30, 2219-2224), (Bal et al., (1994) J.Clin.Micro. 32, 1894–1898), Brown et al., (1995) J.Med.Microbiol, 43,
110-114), Sun-Ho Kee et al., J.Clin.Mico 32, 1035 –1039)..... The comments of the other reviewer reaffirm the value of the PCR in the clinical environment over other conventional PCR methodologies.