Author's response to reviews

Title: Rapid semi-automated quantitative multiplex tandem PCR (MT-PCR) assays for the differential diagnosis of influenza-like illness

Authors:

Elektra Szewczuk (keith.stanley@ausdx.com.au)
Kiran Thapa (kiran.thapa@swahs.health.nsw.gov.au)
Terry Anninos (terry.anninos@imvs.sa.gov.au)
Kenneth McPhie (ken.mcphie@swahs.health.nsw.gov.au)
Geoff Higgins (geoff.Higgins@imvs.sa.gov.au)
Dominic E Dwyer (dominic.dwyer@wmi.usyd.edu.au)
Keith K Stanley (keith.stanley@ausdx.com.au)
Jonathan R Iredell (jiredell@usyd.edu.au)

Version: 4 Date: 10 January 2010

Author's response to reviews:

Further response to Editor BMC Infectious Diseases
MS: 1172942562270580 - Rapid semi-automated quantitative multiplex tandem PCR (MT-PCR) assays for the differential diagnosis of influenza-like illness

Thank you for the opportunity to further improve the MS. Detailed responses as follows:

REVIEWER 1 (Dr Harder):

1. …reasons why a real-time RT-PCR has not been used as a standard diagnostic tool have been left out…
2. …principal concern about lack of transparency in primer targets…
3. …how many strains have been tested?
4. …how about H1N1v?
5. …should include a comment …"descriptive study…..etc"
6. …is the new assay alluded to for H1N1v an MT-PCR? Can we expect it to be useful on the basis of alignments?

R1.1: (RT-PCR…?)

RT-PCR was in fact used as the primary comparator. This was described in Methods (DNA handling and sequencing):"…a nested reverse transcriptase real-time polymerase chain reaction (RT-PCR) was used..." and references were given. We specified this subsequently as "PCR" or "ordinary PCR" in the text in order to clearly distinguish "RT-PCR" from "MT-PCR" (which is also a nested real-time reverse transcriptase PCR, ie an RT-PCR). However, we agree with R1 this seems to have reduced clarity rather than improved it.
ACTION:

1. we have changed all these to "RT-PCR".

2. we also now refer to a comparison of the assay including the "swine flu" target with other rapid diagnostics including another specific in-house RT-PCR in the Discussion (see R1.4/1.6, below). This was submitted and published while this MS was in review and the data could not therefore have been included.

R1.2: (...primer sequences?):

We agree with R1 about the value of the primer sequences. However, we are moving in this country to meet ISO standards for clinical diagnostics over the next 4 years and to phase out "in-house" assays in general (http://www.tga.gov.au/docs/html/devguid1.htm).

Many of the companies taking on this burden wish to protect their intellectual property in the interim by non-disclosure. Available assays from major biotech companies eg Cepheid (a range of assays) and Roche (Septifast) withhold the sequences of their optimised primer sets. Such assays still need clinical evaluation, and our compromise is to state that primers were 100% compatible with specified sequences and to then test the assay in real working laboratories. Many leading journals accept this approach (eg J Clin Microbiol. 2008;46(9):3021--; J Med Microbiol. 2008;57(Pt 5):601--; Eur J Clin Micro Infect Dis 2009; 28(6):659--).

ACTION (1.2):

The authors support Reviewer 1's point of principle, and accept that this is an editorial policy issue. Text is added to the Discussion (see below, after 1.5):

R1.3: (...strain numbers?)

The numbers of controls and clinical specimens are specified in each section. The implied criticism is that Australian samples in two centres may have included only Australian strains and that this assay may not perform well in other parts of the world. We accept that this is even more important when primer sequences are not available (see also comments above - R1.2) and we specifically respond to this as well (below).

ACTION (1.3):

Text is added to highlight this in Discussion (see end of response below, after 1.5):

R1.5: (...descriptive study)

This study follows previous publication of the methodological details, the primary and most comprehensive description of which is specifically referenced (#9), and our study is intended to evaluate performance in two working laboratories using real clinical material. We accept that his has the disadvantage of being unable to control for variable such as specimen quality etc., but it does have the advantage of describing true performance characteristics in the situation for which it is actually designed.
ACTION (1.5):
Text is added to the Discussion (also 1.2 and 1.3), as follows:

(p11)
"This evaluation does not control for specimen variation in specimen quality or operator performance or any of a number of other variables applicable in real working laboratories.

and

(p14)
"However, actual primer sequences may fail to recognise new viral variants and so a lack of transparency by manufacturers in releasing such information requires evidence that the very latest sequences available are completely homologous with the primers included, if clinical laboratories are to trust the assays. Given the ready availability of influenza A sequences (eg. via PubMed), it is reasonable to expect this as a minimum standard. Our testing of fully-characterised strains included only those listed in Table 2, and the reference sequences used are listed in Table 1. In this study, the isolates tested in the clinical laboratories came only from two Australian population centres and different regions may encounter strains which are significantly different from those listed in Tables 1 and 2."

R1.4 and 1.6: (…H1N1v - "swine flu")

As previously stated, the new variant H1N1 came to light for the first time after submission of this MS in March 2009. A newly available assay for H1N1/09 is a minor modification of the assay described herein, as specified in the last amendment. We have now had some direct experience with that assay configuration, and we cite some reports.

ACTION (1.4/1.6):
Text in the Discussion has been amended, with appropriate references, as follows:

(p14,15)
"Since this assay was developed, we have also experienced the influenza A/H1N1/09 pandemic, which caused a significant number of intensive care admissions and deaths in this country (33) and required modifications to include the NP gene of A/H1N1/09 and an M gene consensus (H1N1; A/H1N1/09; H3N2; H5N1). Performance in the Australian 2009 influenza season appears comparable to a nested RT-PCR specifically optimised for A/H1N1/09 only [9, 10]."

REVIEWER 2 (Dr Claas):
1. …a multiplex PCR or a combined set of reactions on the same target?
2. …responses to q4 do not answer the question of whether discrepant results could be contamination…a second confirmatory PCR on another target would be proof…

R2.1: (a true multiplex…?)
The assay system has been described in detail previously (reference #9).

ACTION (2.1):
We have added to the text in the opening sentence of the section headed "Multiplexed tandem PCR (MT-PCR)", which is now as follows:

"This is a two-step assay using nested primer pairs in which the first step involves a highly multiplexed reaction to pre-amplify multiple targets for (typically, 15) cycles. These are then aliquoted into individual reaction tubes containing nested specific PCR primers as template for the second step reaction [11] which was performed using a liquid handling robotics system provided by AusDiagnostics Pty Ltd. (Sydney, Australia)."

R2.2: (…does not answer question re contamination…)
(original answers to q4 were:
Failure to subtype six strains is mentioned early on, in 5 of 6 of which there was evidently very low levels of virus (p.8.: “Six of these could not be assigned a specific haemagglutinin subtype, and most of these latter (5/6) had a normalised INF-A value <30.”). We have now added a reminder that these specimens were all retrieved from storage and template degradation was a significant risk. This section now reads:

“All of these were retrieved from long-term storage, and template degradation was a significant possibility. Six of these could not be assigned a specific haemagglutinin subtype, and most of these latter (5/6) had a normalised INF-A value <30.”

re melt temperatures for specificity: it was stated clearly in Methods that verification was by Agilent Bioanalyser to confirm that the second-stage amplicon was discrete and correct-sized (p 5.) We have now added an additional reference and an additional comment about the melt temperature. The section on p5 now reads:

“The melt temperature, with a range of 1.5oC either side of predicted Tm, was deemed acceptable if the melt curve was normal sigmoidal. Verification of correct-sized discrete amplicons derived in step 2 MT-PCR assays was performed using a Bioanalyzer DNA separation chip (Agilent Technologies, Forest Hill, Victoria, Australia), as previously described [10].”

(and:
A sentence has been added on p12: “No multiple or unexpected-size amplicons were detected, and no discrepant melt Tms were detected; no sequencing of
clinical isolates was performed after initial test validation."

P9 has also been altered and a sentence added for clarification. This now reads (new text underlined):

“The additional INF-A detections were all low-level (normalised result <100) but all had correct-sized discrete second-stage amplicons as determined on Bioanalyzer (Agilent, SantaClara, CA) with sigmoidal cycling curves and single melt peaks and are thought to represent true positive results. Sequencing was performed on six of the unexpected positives to confirm that the Bioanalyser was a reliable guide.”

R2 points out that contaminating first (or second-step) amplicons in any run can never be fully excluded without a repeat independent confirmatory test on an alternate target – we agree that this shortcoming should be highlighted clearly..

ACTION (2.2):
Text has amended in the Discussion, as follows:

"This evaluation does not control for variation in specimen quality or operator performance or any of a number of other variables applicable in real working laboratories. We used an automated liquid-handling system and robotic cycler that operates on a preset algorithm for both cycling and for calling of results. All results were manually verified in this study but required operator expertise is otherwise minimal. Second-stage amplified product is sealed and discarded unopened. Every second-step 72-well gene disc has 12 wells for each target of a 6-target assay, all separated spatially. The additional positives we describe came on different days, all with negative controls and with several other negatives in the runs in which they were detected. All MT-PCR reactions yielded normal melt curves and all reactions giving unexpected results also contained discrete correct-sized amplicons. No multiple or unexpected-size amplicons or discrepant melt temperatures were identified. Nevertheless, sequencing of clinical isolates was not performed after initial test validation and no additional alternative independent testing was performed to specifically verify second-step results, so contamination of the second-step reaction with unrelated first-step amplicons cannot be completely excluded."

In order to save space in Discussion, which was already overlength, we have deleted some phrases and sentences, mostly about H5N1. The total Discussion length is now 1422 words; previously 1566.

We hope that this version is now acceptable and thank the reviewers for their patience.

Jon Iredell