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 (jon.iredell@swahs.health.nsw.gov.au)

Version: 2 Date: 20 October 2009

Author's response to reviews: see over
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 respond to comments. Please note that there have been significant delays due to web access problems which have last week been resolved.

With respect to issues raised by Reviewer 1 (Harder):

Major revisions:

1. *lack of transparency in primer targets...
   a. The reviewer requests a specific statement be made. This information is commercial-in-confidence and the same comment is now inserted as was used in our description of the yeast assay in J Clin Microbiol 2008; 46(9):3021-3027: “Primer and artificial internal control sequences are not shown due to commercial confidentiality agreements with AusDiagnostics Pty Ltd. The target regions are specified in Table 1, as are example sequences. The internal control is a contrived sequence that does not appear in nature. No base pairing redundancies were specified in any of the primers used” (p5)
   b. The target regions and sequences are specified in greater detail in Table 1, as requested; no primer redundancies were used (– see above)

2. *low numbers of reference strains...

The reference strains are stated in the text of the Methods section (p 4: Clinical and control specimens) and were therefore not presented in Table form. We would be happy to do so but reasoned that testing of reference strains with known sequences add little or nothing to the list of strains against which nucleic acid alignments were tested (see Table 1), and that our principal aim was to conduct a test of performance in two real clinical laboratories.

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.”

*remelt 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 1.5°C 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].”

3. ...use of different sample cohorts...
The reviewer asks us to make it obvious that two different sample cohorts were used to compare MT-PCR with other standard techniques. We have therefore inserted a phrase (new text is underlined) on p10 to make this more apparent, as follows:

“Comparison of MT-PCR assays with standard PCR.
One hundred and seventy-six additional clinical specimens, completely unrelated to those tested above, were tested by PCR at the IMVS:…”

4. ...statistics...
These assays were conducted in two different laboratories, in two different locations, in two different cohorts of real clinical specimens. We conducted this as a descriptive study and did not control for specimen variation in quality, quantity, operator performance or any of a large variety of confounding variables. For this reason, we report the results in descriptive fashion and gave simple predictive values as the most straightforward guide to actual performance in two working laboratories.

We are prepared to provide basic statistical analyses but did not do so as we believed this may misrepresent the study as highly controlled. We note that the second reviewer did not believe that statistical analyses were needed.

5. “...no mention of H1N1 SIV...
The first reported death from influenza A/H1N1/09 and the first reported case outside Mexico was at the very end of April 2009, more than a week after this manuscript was submitted. At this stage, very little was known about the virus and no sequences were available. This assay has since been adapted to manage the A/H1N1/09 outbreak in Australia but clinical performance data have not yet been published. We have added a sentence (and one reference) in the Discussion, as requested:

“Since this assay was developed, we have experienced the influenza A/H1N1/09 pandemic, which caused a significant number of intensive care admissions and deaths in this country (40). An assay targeting the NP gene of A/H1N1/09 and an M gene consensus (A/H1N1/09; H3N2; H1N1), is now available (http://www.ausdiagnostics.com), but clinical performance characteristics have not yet been described. “

Minor revisions:
1. move passage from Methods, Clinical and control specs to MT-PCR : done
2. Immunofluorescence: We have added in a reference for IF testing. This section now reads:
   “Immunofluorescence was performed using fluorescein-conjugated monoclonal antibodies (Chemicon International, Temecula, CA, USA) against influenza A and B, RSV, adenoviruses, and hPIV1-3 on acetone-fixed smears of material from respiratory tract specimens, as indicated and as previously described [11].”
3. MT-PCR control: see 1a, above.
4. **Results: Assay Development** – as requested, this has been moved to the Methods section, and now appears immediately above the Results heading rather than immediately below it.

5. **Copy number of fluA** – determined in the reference laboratory by serial dilutions to determine number of plaque-forming units (pfu)/mL. The key reference was given (Clinical and Control specimens, and Table 2 footnote – ref #10), but we have now altered the sentence on p.4 for clarification (new text underlined):

   “Gamma-irradiated MDCK-tissue culture extracts with known amounts of Indonesian (Indo 05, Indonesia "clade 2") and Vietnamese (HN 3028, Vietnam "clade 1") strains of H5N1 influenza A virus were provided Australia-wide as control templates [10], and we used these along with locally cultivated tissue culture extracts of H3N2 A/Fujian/114/2002-like and H1N1 A/New Caledonia/20/99-like Influenza A virus and B/Shanghai/361/2002 Influenza B virus.”

6. **Results: Has sequencing been done?**

   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.”

7. **What are the standard PCRs used?**

   The PCRs in use are as previously described, and a reference is given in Methods (the text reads:

   “A nested reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect influenza A and B, as previously described [13].”).

   We have amended this to include another reference specific to the IMVS laboratory, so that the relevant sentence now reads:

   “A nested reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect influenza A and B [13] and other respiratory viruses [14] , as previously described.”

8. **Table 3 – corrected.**

With respect to issues raised by **Reviewer 2** (Claas):

**Major comments:**

1. **...H1N1/09...**

   See response to Reviewer 1 (#5), above

2. **Why develop quantitative assays for respiratory tract infection?**

   The value of accurate monitoring of declining viral load is well proven in almost many therapeutic contexts (e.g., HIV, CMV, Hepatitis viruses B and c) and we argue (with Table 5) that this is no less valid for respiratory viruses.
3. *p8 – why aren’t all the institutional samples the same subtype?*
Co-infection and co-circulating virus is common in the normal influenza season and we believe this illustrates the value of multiplexing and of more specific testing, both available in this assay. We therefore add some new text (underlined) and another reference to our closing argument in the Discussion, to say: 
” Importantly, this method simultaneously subtypes Influenza A, identifies multiple pathogens and co-infections in a single specimen and provides potentially valuable quantitative data and should be regarded as a new benchmark for respiratory virus diagnosis. A multiplexed method also facilitates recognition of co-circulating viruses in outbreak situations in which they might otherwise be assumed identical [42].”

4. *Discrepant results (p.8 and 9) …contamination a possibility?*
See responses to Reviewer 1 (major #2 and minor #6), above.

5. *why wasn’t hPIV-3 added to the multiplex?*
The MT-PCR assay is configured most efficiently as factors of 72. Adding one reaction to the multiplex decreases second-stage efficiency (6x12=72 vs 7x10=70) and occurs for the sake of including an assay (HPAI H5N1) which was irrelevant in the Australian influenza season from which these specimens were extracted. These details are explained in the references only, and so the text has now been altered on p. 6-7 for clarification, as follows (new text underlined):
” Assay development. 

Assays were developed to detect Influenza viruses A and B (INF-A and INF-B), the Respiratory Syncytial Virus (RSV), Rhinovirus (RV), and human Parainfluenza virus type 3 (hPIV-3), and to identify Influenza A haemagglutinin gene types H1, H3 and H5 within a multiplexed tandem PCR (MT-PCR) assay profile [9], using sequences available from strains in our laboratory and lodged in GenBank, including the reference sequences listed in Table 1. Multiplexed assays are generally configured so as to divide neatly into 72 (the number of positions available in a standard step 2 template in the RG6000 cycler), with no empty wells remaining. Primers targeting the Influenza A haemagglutinin H5 gene (in “Influenza profile 1”) were replaced by primers targeting the nucleoprotein gene of human Parainfluenza virus type 3 (hPIV-3) in another assay (“profile 3”).”

The revised manuscript, containing all alterations as specified above, has been uploaded.

Yours sincerely

Jon Iredell