Reviewer’s report

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

Version: 1 Date: 29 July 2009

Reviewer: Eric C.J. CJ Claas

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This manuscript describes a novel approach of multiplex PCR for diagnosing respiratory tract infections, with particular attention for influenza. Interesting is the fully automated assay set up, which brings molecular diagnostics a step further towards becoming point-of-care testing.

Obviously, the recent developments with pandemic influenza H1N1 2009, prompt the immediate question how easily this new target is included in the designed assay.

Main concern of the assay is the fact that the assay is based on a nested PCR approach, which makes the assay more sensitive, but also more prone to contamination issues, despite the automated liquid handling. One important question is if there is a clinical need for increased sensitivity in RTI testing. In acute infections there usually is sufficient virus present to be detected by (non-nested) PCR assays. Is detection of a single copy of a virus clinically relevant?

Culture positive samples in real-time PCR in general have Ct values < 32, but can be as low as Ct=20. A pre-amplification step in these cases will result in lots of amplicons being transferred to the nested amplification.

Further comments
- in some seasons, one billion cases may occur, but not every year. It is not in the factsheet either.
- The recent new pandemic H1N1 should be included, preferentially in the assay, but at least in the discussion.
- why is it necessary to develop quantitative assays for respiratory tract infections? The Ct value in real-time PCR provides an good indication.
- Page 8: If the 18 infA positive samples came from an institutional outbreak, shouldn’t all have the same hemagglutinin subtype?
- The discrepant results (page 8 and 9) without explanation by previous positive samples should be analyzed in more detail. Is contamination a possibility?
- Page 5: The H5 assay was replaced by hPIV-3. Why wasn’t this assay added to the multiplex?

Level of interest: An article of importance in its field
Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests