**Critical evaluation of the methodology used by Wilson-Davies *et al.,* (2020)entitled *“*Concerning the OptiGene Direct LAMP assay, and it`s use in at-risk groups and hospital staff”**

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Dear Editor-in-Chief,

As part of a multi-institution pilot project across various sites in England and Gibraltar, on the 1st of December, in conjunction with NHS Test and Trace and the Department of Health and Social Care (DHSC) we published the diagnostic sensitivity (DSe) and specificity (DSp) of the OptiGene Direct PLUS RT-LAMP assay (OptiGene) on both swabs and saliva1.

We write with concern regarding the recent letter from Wilson-Davies *et al.* who have carried out an evaluation of the performance of the OptiGene Swab Direct RT-LAMP assay against the CDC N1/N2 SARS-CoV-2 RT-qPCR and have used that data to suggest that the OptiGene Direct Saliva PLUS RT-LAMP assay is significantly less than published1.

Firstly, the authors citeFowler *et al.*2 as the publication of topic in the letter. This is not the assay format evaluated by the Test and Trace/DHSC pilot for wider application1. The Direct RT-LAMP assay described in the Fowler *et al.* publication2 was for use on swabs and was superseded with a new sample preparation method (Direct PLUS RT-LAMP assay) to enable the Direct RT-LAMP assay to be performed on swabs and saliva. It was the Direct PLUS RT-LAMP assay that was in use at all the sites which participated in the studies reported in the NHS Test and Trace and the DHSC report1.

Secondly, Wilson-Davies *et al* state that the Direct PLUS RT-LAMP assay “failed to detectmore than 50% of saliva positive cases in a pilot in Greater Manchester” and use the Guardian newspaper as their source of evidence3. The Guardian reported, out of context, a subset of data selectively extracted from within the Test and Trace/DHSC pilot1. Notably, the data generated by the Manchester Regional Virus Laboratory team, contrary to the statement by Wilson-Davies *et al*, verified the test performance, achieving 100% DSp and 100% DSe (CT <25), well within the confidence intervals reported in the government publication, which includes the Manchester pilot data1. The Manchester subset analysis included 15 positive samples, of which only six had a high-to-medium viral load (CT <25). The nine lower viral load samples (CT >25) artificially skewed the overall sensitivity of the test as the categories were not balanced (exhibiting the full dynamic range in equal proportions of high medium and low CT values).

Thirdly, Wilson-Davies *et al.*state “consistent with results from the Manchester Regional Virus Laboratory, we have been unable to replicate the sensitivity”. However, as we have stated, the Manchester Regional Virus Laboratory produced results that were consistent with the performance of the test within the range of detection for its intended use case1, therefore Wilson-Davies *et al* data is not consistent with the Manchester Regional Virus Laboratory data. In addition, the Manchester Regional Virus Laboratory evaluated the performance of the Direct PLUS RT-LAMP assay on saliva and not on swabs as used by Wilson-Davies *et al*. The performance of a test differs depending on the sample matrix (saliva or swab) being analysed (see table 4 and paragraphs below for further explanation).

Fourthly, Wilson-Davies *et al.* have estimated genome copies (digital copies per milliliter: dc/mL) using the nucleocapsid (N) gene target. The Direct PLUS RT-LAMP assay targets the viral genomic RNA which is only present during active replication. Tests targeting regions of the viral genomic RNA which are not present also as subgenomic mRNAs (e.g., ORF1ab) are the most accurate approximates of actual number of viral genomes present. In contrast, N gene RNAs are present in abundance as subgenomic mRNAs and because of increased copy number, lead to overestimations of the number of actual viral genomes by ∼100-1000 times4. In addition to this, the N gene has been reported by many to persist well beyond ORF1ab4, 5, 6, 7 and is therefore a poor indicator of active replication when present as the only target in the RT-qPCR. The swab samples listed in table 1 therefore, could include samples which were N gene positive and ORF1ab negative. If the same genomic region is not used when comparing different chemistries, the DSe and DSp can be significantly miscalculated. Wilson-Davies *et al.* have previously used RT-qPCR assays including ORF1ab for outbreak management in NHS staff and therefore could have easily made more appropriate calculations and comparisons based on the use case of this test which is to identify individuals who are likely to be infectious8. Nevertheless, if the data by Wilson-Davies *et al.* is adjusted in line with the likely overestimation of genome copies calculated from using N gene4 (Table 1), the analytical sensitivity is representative of the Direct PLUS RT-LAMP assay reported performance (< 103)1, 2.

|  |  |  |  |
| --- | --- | --- | --- |
| SARS-CoV-2 Viral load estimated using CDC N1/N2 PCR | Number of positive samples | Adjusted dc/mL100X overestimation | Adjusted dc/mL1000Xoverestimation |
| 1, 000 - <10,000 | 103-104 | 0/18 | 101-102 | 10-1-101 |
| 10, 100 - <100,000 | 104-105 | 0/20 | 102-103 | 101-102 |
| 100,000 - <1, 000,000 | 105-106 | 6/17 | 103-104 | 102-103 |
| <1, 000,000 - <10,000,000 | 106-107 | 18/25 | 104-105 | 103-104 |
| >10,000,000 | 107-108 | 5/6 | 105-106 | 104-105 |

**Table 1.** Analytical/Diagnostic sensitivity reported by Wilson-Davies *et al.,* with the dc/ml adjusted for overestimation of actual viral genome copies.

We have further independently explored the analytical sensitivity of the Direct PLUS RT-LAMP assay using UV treated virus as this causes much less fragmentation to RNA when compared to chemically and heat inactivated virus such as the NIBSC blinded panel as reported 1. The analytical sensitivity of the Direct PLUS RT-LAMP assay was determined to be between 1,000 and 100 plaque forming units [PFU]/ml (0.5-2 PFU/reaction) (Table 2) which translates to 100-1000 copies of infections virus/ml given that one PFU represent progeny derived from one infectious virion. This sensitivity is consistent with the previous publications1 and further emphasize the methodological problems in the conclusions drawn by Wilson-Davies *et al* in their original table 1.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| PFU/ml | PFU/Reaction  | Replicate 1 | Replicate 2 | Replicate 3 |
| 100,000 | 500 | Positive | Positive | Positive |
| 10,000 | 50 | Positive | Positive | Positive |
| 1,000 | 2 | Positive | Positive | Positive |
| 100 | 0.5 | Positive | Negative | Positive |
| 10 | 0.05 | Negative | Negative | Negative |
| 1 | 0.005 | Negative | Negative | Negative |

**Table 2**. Analytical sensitivity using UV inactivated SARS-CoV-2

It is also important to note that the swab samples used for analysis by Wilson-Davies *et al.* had not been tested fresh but had been stored at 4oC for up to 36 hours prior to being frozen at -80oC. The Direct PLUS RT-LAMP assay has been validated for use on fresh, non-freeze thawed samples (saliva and swabs) and there is a dedicated sample collection standard operating procedure (SOP) to reflect this requirement. Wilson-Davies *et al.* did not collect and store their samples according to this SOP.

To understand the effect of freeze thaw on success rates and to inform the SOP, we analysed twenty positive saliva samples from asymptomatic individuals detected by Direct PLUS RT-LAMP and confirmed by RT-qPCR when performed on freshly collected, non-freeze thawed samples. These were re-analysed in triplicate after the sample had been frozen for at least a week and then received one freeze thaw (Table 3). Only eight of the twenty samples remained positive in all replicates following freeze thaw. This highlights the considerable effect of freeze thawing on the performance of the Direct PLUS RT-LAMP assay due fragmentation of RNA (LAMP amplifies much larger fragments when compared to RT-qPCR and is therefore susceptible to sample degradation). Whilst this stability data was performed on saliva, we would anticipate the same effect on swabs since the process of degradation by freeze thaw is no different between sample types when considering LAMP based amplification. Given that all samples used in table 1 of Wilson-Davies *et al.* were freeze thawed prior to analysis the data is not representative of samples which would be used in the designated use case of this assay. Storing samples at 4oC was also more greatly affected than those stored at room temperature, which is also what Wilson-Davies *et al.* did prior to freezing. We also assume that the swabs used were nasopharyngeal/orophayngeal collected in VTM which is a IFU requirement. Any other diluent or type of swab (including anterior nasal swabs) would also impact the performance.

|  |  |  |
| --- | --- | --- |
|  | Fresh | One freeze thaw |
| Sample | RT-qPCR  | LAMP 1 | LAMP 2A | LAMP2B | LAMP 2C |
| 1 | 20.07 | **POSITIVE** | **POSITIVE** | **POSITIVE** | **POSITIVE** |
| 2 | 20.25 | **POSITIVE** | **POSITIVE** | **POSITIVE** | **POSITIVE** |
| 3 | 20.31 | **POSITIVE** | **POSITIVE** | **POSITIVE** | **POSITIVE** |
| 4 | 20.35 | **POSITIVE** | **POSITIVE** | **POSITIVE** | **POSITIVE** |
| 5 | 20.93 | **POSITIVE** | **POSITIVE** | **POSITIVE** | **POSITIVE** |
| 6 | 23.08 | **POSITIVE** | NEGATIVE | **POSITIVE** | **POSITIVE** |
| 7 | 23.33 | **POSITIVE** | **POSITIVE** | **POSITIVE** | **POSITIVE** |
| 8 | 23.61 | **POSITIVE** | **POSITIVE** | **POSITIVE** | **POSITIVE** |
| 9 | 23.93 | **POSITIVE** | **POSITIVE** | **POSITIVE** | **POSITIVE** |
| 10 | 24.08 | **POSITIVE** | NEGATIVE | **POSITIVE** | **POSITIVE** |
| 11 | 25.7 | **POSITIVE** | NEGATIVE | **POSITIVE** | NEGATIVE |
| 12 | 26.4 | **POSITIVE** | NEGATIVE | NEGATIVE | **POSITIVE** |
| 13 | 27.47 | **POSITIVE** | NEGATIVE | NEGATIVE | NEGATIVE |
| 14 | 27.49 | **POSITIVE** | NEGATIVE | NEGATIVE | NEGATIVE |
| 15 | 27.60 | **POSITIVE** | **POSITIVE** | NEGATIVE | NEGATIVE |
| 16 | 27.62 | **POSITIVE** | NEGATIVE | NEGATIVE | NEGATIVE |
| 17 | 28.14 | **POSITIVE** | NEGATIVE | **POSITIVE** | **POSITIVE** |
| 18 | 28.14 | **POSITIVE** | NEGATIVE | **POSITIVE** | NEGATIVE |
| 19 | 28.22 | **POSITIVE** | **POSITIVE** | **POSITIVE** | NEGATIVE |
| 20 | 28.89 | **POSITIVE** | NEGATIVE | NEGATIVE | NEGATIVE |

**Table 3.** Effect of one freeze thaw on the ability to detect SARS-CoV-2 by Direct RT-LAMP assay

Wilson-Davies *et al.*state “A sample of >10,000,000 (>107) digital copies/mL (dc/mL), collected from a symptomatic NHS staff member, swabbed on day one of symptoms, produced a negative result when tested by the OptiGene Direct LAMP method. Similarly, a swab from an asymptomatic staff member, a target group for this assay, with 2,500,000 (2.5x106) dc/mL was missed. A false negative result, with a high viral load, is a patient safety risk, particularly if the sample belongs to an asymptomatic member of staff”. Taking the adjusted dc/ml from Table 1, these two samples would be: symptomatic: [104-106]andasymptomatic [103-105] which are well within the range of detection and also detected in other samples by this team. Explanations for the above result include: a) the effect of freeze thawing which would have no effect on RT-qPCR detection, but profound effects on RT-LAMP detection (as described above), or b) the technical performance of the operator. NEQAS verification for technical performance of this assay is a prerequisite to ensure consistent and expected performance before the assay is used on clinical samples. We have worked with NEQAS to construct a validation panel that all sites must utilise before running the Direct PLUS RT-LAMP assay and has been validated by multiple independent sites. This panel will be listed on the NEQAS website in due course. Unfortunately, Wilson-Davies *et al.*have to date not participated in this quality assurance programme and therefore cannot guarantee or substantiate the technical performance of the assay in their hands.

Fifthly, this laboratory had previously reported to two signatories on this letter (Fowler and Kidd, personal communication) an inability to reproduce sensitivities expected for the RNA RT-LAMP assay. A training visit was made to this laboratory in August 2020, and several critical observations were constructively made, the most significant of which was lack of mixing of the mastermix and the RNA sample prior to use. During that visit, a number of samples were provided by this group’s laboratory and were run under the supervision of RT-LAMP assay experts. With proper mixing of mastermix and RNA, at this time, this laboratory produced the expected performance of the assay as agreed by the laboratory team at the time. No such training has ever been provided for the Direct PLUS RT-LAMP assay protocol. Critically the Direct PLUS RT-LAMP assay mix is much more viscous than the RNA RT-LAMP assay mix so if the same behaviours observed during the training visit for RNA RT-LAMP remained during the performance of the Direct PLUS RT-LAMP assay, performance of the assay will have been compromised.

Sixthly, when working on clinical samples there is a lysis and heat step which needs to be performed with no deviation from the IFU and dedicated bench card. This laboratory has not approached the UK quality assurance leads for LAMP testing for the bench card or IFU, which may explain a loss of performance due to technical error.As mentioned above the Direct PLUS RT-LAMP swab assay must also be performed using nasopharyngeal or oropharyngeal swabs collected into VTM.

Seventhly, Wilson-Davies *et al.*state “It has been asserted that nucleocapsid protein (N-) gene targets for qPCR are not valid for assessment of the performance of the OptiGene direct LAMP assay. The reason provided is that N-gene qPCR detects sub-genomic mRNA that may persist, and be detectable, beyond the period of viral replication, with an inability to differentiate between RNA from replicating virus and residual RNA after a resolved infection. It is important to stress that duration of detection of sub-genomic mRNA, and its implications for infectiousness, are currently debated”. This statement of Wilson-Davies *et al.,* is factually incorrect. The replication cycle of SARS-CoV-2 is well defined fundamental virology, not a matter of debate9. Samples have been shown by multiple different authors to remain positive for N gene for several days/weeks/months after targets on the ORF1ab viral genomic RNA are no longer detected 4, 5, 6, 7. Given that Wilson-Davies *et al.,* have used RT-qPCR assays containing the ORF1ab (which also includes N as a different target) for outbreak management in NHS staff, it is surprising that this team would choose to use the CDC N1/N2 RT-qPCR as a single gene mis-matched comparator which is likely to have overestimated actual viral genomic copies present in the sample.

Eighthly, Wilson-Davies *et al.*state“[saliva has] reduced sensitivity in comparison to the higher sensitivity of a nose and throat swabs” without presenting any new data on saliva-based testing. In raising this topic, Wilson-Davies *et al.* do not provide a balanced reference to an ongoing debate, which includes published reports demonstrating high RT-qPCR test concordance between appropriately collected paired saliva and nasopharyngeal samples 10, 11.

To evaluate whether there was a difference in detection of SARS-CoV-2 in swabs compared to saliva a panel of 26 paired samples (52 samples) provided blinded by the Milton Keynes Lighthouse laboratory were analysed by an RT-qPCR (including ORF1ab) and Direct PLUS RT-LAMP on both saliva and swabs. The saliva and swabs had been collected from the same individual (Table 4). Eight samples were detected only in swabs or saliva with the remaining 10 samples detected in both swabs and saliva. Fifteen samples had viral loads (lower CTs) which were higher in saliva when compared to the paired swab. Ten samples had viral loads (lower CTs) which were higher in swabs when compared to the paired saliva and one sample had comparable viral loads. This is consistent with the publication of Hansen *et al* 12*.,* who reported that no single specimen type detected all SARS-CoV-2 infections, but that nasopharyngeal swabs (n = 80) and saliva (n = 81) were comparable and superior when compared to that of anterior nasal swabs (n = 70). In is inaccurate and misleading of Wilson-Davies *et al* to therefore claim that saliva is inferior to swabs. In fact in a recent preprint, viral load in saliva but not nasopharyngeal swabs has been shown to be a dynamic unifying correlate of COVID-19 disease presentation, severity and mortality13.

|  |  |  |
| --- | --- | --- |
|  | Swab | Saliva |
|  | **Direct RT-LAMP** | **RT-qPCR ORF1ab** | **Direct RT-LAMP** | **RT-qPCR ORF1ab** |
| 1 | **POSITIVE** | 24.7 | NEGATIVE | 25.38 |
| 2 | **POSITIVE** | 21.72 | NEGATIVE | 26.14 |
| 3 | **POSITIVE** | 21.52 | NEGATIVE | 25.29 |
| 4 | NEGATIVE | 27.87 | **POSITIVE** | 25.33 |
| 5 | NEGATIVE | 30.42 | **POSITIVE** | 25.35 |
| 6 | NEGATIVE | 30.33 | **POSITIVE** | 25.2 |
| 7 | **POSITIVE** | 26.59 | NEGATIVE | 29.16 |
| 8 | **POSITIVE** | 26.51 | NEGATIVE | 30.17 |
| 9 | NEGATIVE | 30.39 | **POSITIVE** | 26.48 |
| 10 | **POSITIVE** | 28.49 | **POSITIVE** | 25.96 |
| 11 | **POSITIVE** | 22.72 | **POSITIVE** | 19.15 |
| 12 | **POSITIVE** | 22.49 | **POSITIVE** | 19.02 |
| 13 | **POSITIVE** | 22.67 | **POSITIVE** | 24.1 |
| 14 | **POSITIVE** | 22.63 | **POSITIVE** | 24.18 |
| 15 | **POSITIVE** | 25.67 | **POSITIVE** | 15.64 |
| 16 | **POSITIVE** | 24.87 | **POSITIVE** | 15.3 |
| 17 | **POSITIVE** | 21.51 | NEGATIVE | 26.64 |
| 18 | **POSITIVE** | 21.24 | NEGATIVE | 24.09 |
| 19 | NEGATIVE | 31.25 | **POSITIVE** | 27.89 |
| 20 | **POSITIVE** | 21.86 | **POSITIVE** | 22.04 |
| 21 | **POSITIVE** | 21.65 | **POSITIVE** | 21.93 |
| 22 | NEGATIVE | 29.34 | **POSITIVE** | 24.07 |
| 23 | NEGATIVE | 29.43 | **POSITIVE** | 23.87 |
| 24 | **POSITIVE** | 30.93 | **POSITIVE** | 24.42 |
| 25 | NEGATIVE | 30.53 | **POSITIVE** | 23.5 |
| 26 | **POSITIVE** | 29.21 | NEGATIVE | 28.79 |

**Table 4.** Performance of Direct RT-LAMP on blinded and paired saliva and swab samples from lighthouse laboratory samples

Ninthly, Wilson-Davies *et al.*state ***“***Critically, the inclusion of an internal control, added before nucleic acid extraction, is a vital quality requirement for molecular diagnostic work”. The Medicines and Healthcare Products Regulatory Agency (MHRA) target product profile (TPP) for SARS-CoV-2 molecular assays says that an internal control is desirable, but the availability of one as a separate inclusion is acceptable14. OptiGene have a range of human RNA controls compatible with RT-LAMP assays which sites can choose to use if desired.

Finally, the Direct PLUS RT-LAMP assay requires fresh reagents that have not been allowed to deteriorate as per the IFU for this assay. Wilson-Davies *et al.*have never ordered reagents from OptiGene so it remains unclear where their reagents were sourced from.This raises significant concern about the provenance, storage conditions, shelf-life and the version & exact type of LAMP reagents used in this study which may account for the apparent deterioration in performance reported in the letter from Wilson-Davies *et al.*

In conclusion, we have highlighted a series of methodological concerns with the laboratory evaluation carried out at the group’s laboratory. Novel diagnostic technologies require different ways of working compared to conventional virology technologies (i.e., RT-qPCR) and if precise attention to detail is not paid to the IFU this may lead to methodological errors such as in this dataset. The use case for this test is to identify asymptomatic/presymptomatic/symptomatic infectious individuals as a surveillance or case finding tool and not as a diagnostic test. Without this type of assay the UK will likely fall short of providing the diagnostics capacity it so desperately needs.

We look forward to hearing from you

Yours Sincerely



Dr Veronica Fowler

Signed on behalf and with agreement of all authors

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