Tuesday, 9th of June 2020

Public Health Laboratory Network Statement on Nucleic Acid Test False Positive Results for SARS-CoV-2

Diagnostic testing for SARS-CoV-2 (the virus that causes COVID-19) is central to suppressing and containing the COVID-19 pandemic in Australia. Nucleic acid tests (NAT), predominantly reverse transcriptase polymerase chain reaction (RT-PCR) are the primary diagnostic tests used for SARS-CoV-2 detection in Australia and are the benchmark for acute diagnosis of COVID-19 infection. RT-PCR tests are very accurate, however false positive and false negative results can occur on rare occasions.

False positive tests occur when a positive test result is issued from a laboratory in the absence of disease. Some potential causes of false positive test results include mislabelling of specimens, data entry errors, contamination of the primary specimen, misinterpretation of a test result, or off-target test reactivity caused by unsuitable testing platform set up or use (see Appendix A for further detailed discussion). Possible sources of false positivity are also further described in Table 1 at Appendix B.

PHLN emphasises that the likelihood of false positive results occurring is very low. Australian laboratories performing NAT testing are required to implement the National Pathology Accreditation Advisory Council's Requirements for Medical Testing of Microbial Nucleic Acids quality framework, which includes procedures to minimise the risk of false positives tests. It should be noted that as community prevalence of COVID-19 falls and the rate asymptomatic testing increases, the proportion of false positive test results may increase slightly. Noting the public health consequences of a false negative result, a false positive result does not have the same ramifications, and on balance, public health action erring on over investigation rather than not investigating is a safer error.

Recommended mitigation strategies to prevent false positive results

PHLN recommends the below mitigation strategies to prevent the occurrence of SARS-CoV-2 false positive results in laboratory settings and clinical/public health impact. Full consideration of available mitigation strategies, including preventative measures and laboratory actions is described in Table 1 at Appendix B.

- Maintain a quality framework in accordance with the National Pathology Accreditation Advisory Council's Requirements for Medical Testing of Microbial Nucleic Acids, which includes procedures to minimise the risk of false positives tests;
- Maintain high standards of quality control at all stages of testing – including pre-analytical, analytical and post–analytical components;
- In a low prevalence setting, samples with a high cycle threshold (Ct) value should be interpreted with caution; and repeat testing of the same sample and re-sampling considered.
- Maintain laboratory protocols and procedures to manage low level amplifications (high Ct values) and/or unexpected positive results, such as;
  - Re-extraction and repeat testing of the same sample using the same assay
  - confirmatory testing of the same sample using a different assays with a different SARS-CoV-2 target (of equal or better sensitivity),
  - patient specimen re-collection and testing.

- Ensure the clinical microbiologist (pathologist) providing clinical governance (including direct liaison with clinicians and public health officers) is available to discuss unusual and/or unexpected cases;
- Ensure close collaboration between the laboratory, local public health units and clinicians to identify potential false positives, ensure clear understanding of test results and potential limitations and, if required, to initiate further testing.

PHLN STATEMENT ON SARS-COV-2 NUCLEIC ACID TEST FALSE POSITIVE RESULTS

Nucleic acid tests (NAT), predominantly reverse transcriptase polymerase chain reaction (RT-PCR) are the primary diagnostic tests used for SARS-CoV-2 detection in Australia.

There are a range of NAT tests used in Australian laboratories, including
- In-house or commercial assays
- Assays that target different parts of the SARS-CoV-2 genome, including the N gene, E gene, RdRp gene and Orf-1 gene
- Single gene target assays, or assays that detect multiple SARS-CoV genes in one test

False positive tests

False positive tests occur when a positive test result is issued from the laboratory in the absence of disease. Table 1 outlines the causes of false positive tests, which can be broadly separated into preanalytical (pre-test) factors, analytical (factors related to test performance such as false-positive reactive RT-PCR) and post-analytical (errors related to incorrect reporting of the result).

In practice, the main categories of false positive reactivity in PCR are (1) laboratory error, and (2) off-target reactivity, of which laboratory error is the most frequent contributor in clinical practice.

(1) Laboratory Error

Laboratory error does not vary with prevalence in the tested population, although it may increase with pressure on laboratories due to episodes of increased throughput. Such episodes may coincide with testing of large numbers of samples with low pre-test probability as has occurred at some stages of the current COVID-19 pandemic. Common categories of laboratory error include: (a) clerical error (b) wrong sample tested (c) cross-contamination from positive samples (d) reagent contamination with amplicon. These are presented in more detail in Table 1.

(2) Off target reactivity
Off-target reactivity is relatively uncommon but is an inherent characteristic of a PCR assay, the relative robustness or otherwise of assay design and of clinical implementation. Off-target reactivity may include (a) cross reaction with non-target genetic material, and (b) self-priming phenomena in the absence of target.

Sources of PCR Off-target Reactivity

A PCR reaction amplifies a highly specific target region of the SARS-CoV-2 genome. SARS-CoV-2 amplicons may be identified by fluorescent probes or by melt curve analysis of intercalating dye. Each amplification reaction is known as a cycle. A usual PCR assay runs for 35-45 cycles. The cycle threshold (Ct) value of a reaction is the cycle number when the fluorescence of a PCR product can be detected above the background signal (Figure 1). Whilst extending the number of cycles beyond 40 may increase the sensitivity of the assay (or ability to detect very low levels of virus in the specimen) it may also increase the risk of false positivity due to non-specific amplification. This may be identified by assessing the Ct value (usually high), shape of the amplification curve and/or performing a melt curve analysis
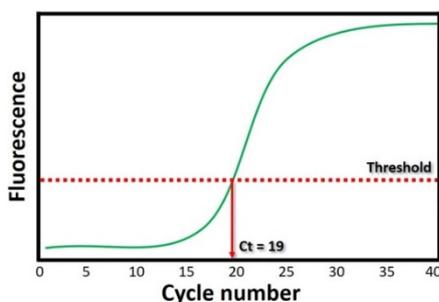

Figure 1: Typical amplification curve of a positive (detected) result

Commercial assays often have in-built analysis systems to interpret a result as detected (positive) or not detected (negative). To comply with TGA requirements, the laboratory must report the results according to the manufacturer's recommendations.

For in-house assays, the parameters for determining a detected (positive) or not detected (negative) result have been determined by the laboratory through the assay development and evaluation process, potentially allowing for greater scope for scientific interpretation and identification and reporting of equivocal / borderline / indeterminate results.

Mitigation of False Positive Reactivity

This is achieved by appropriate PCR design and implementation of a quality laboratory system approach.

PCR assays are extraordinarily high-fidelity assays which are designed for maximal sensitivity and minimal off-target reactivity. They benefit from a design process that includes accurate in silico design of PCR primers and probes and use of extensive databases to identify high-fidelity target sequences with minimal potential off-target interactions. Furthermore, the formats used for PCR enhance fidelity, such as the use of nested, or hemi-nested PCR formats, or formats that include binding of a target specific probe, potentially boosting achievable specificity. The use of multiple gene targets within the same test, or reflex testing of positives samples with a second PCR test using a different gene target also increases specificity.

Cumulatively, all these factors contribute to specificity and make PCR assays the highest fidelity assays used in infectious disease diagnostics. Observed specificity in use bears this out, albeit with certain caveats. For example, one Public Health Laboratory is aware of only two false positives out of the more than 75,000 SARS-CoV-2 PCR tests performed as at 29 May 2020. Taken at face value this equates to a false positive rate of 0.003%.

Within the laboratory false positive results may not always be easily identified, and laboratory staff, clinicians and/or public health physicians should remain vigilant for the presence of false positive results. This may arise from discrepant clinico-epidemiological findings, unexpected laboratory results (such as when a batch of or consecutive samples test positive), erroneous results from external quality assurance programs, warnings from diagnostic companies about potential contaminated assays or reagents, or when supplemental NATs or other diagnostics tests such as genomic sequencing or serology do not concur with the initial NAT result.

As per the National Pathology Accreditation Advisory Council's Requirements for Medical Testing of Microbial Nucleic Acids (second edition 2013), diagnostic laboratories must be aware of the risk of contamination for NATs and have documented procedures for reviewing suspected false positive results. Laboratories must also retain records documenting contamination events that includes the identified source of contamination (if known), and measures taken to reduce the risk of such events in the future.

Further to this, laboratories must:
- Maintain high standards of quality control at all stages of testing – including pre-analytical, analytical and post–analytical components;
- Maintain laboratory protocols and procedures to manage low level amplifications (high Ct values) and/or unexpected positive results, such as;
  - Re-extraction and repeat testing of the same sample using the same assay
  - confirmatory testing of the same sample using a different assays with a different SARS-CoV-2 target (of equal or better sensitivity),
  - patient specimen re-collection and testing.
- Ensure the clinical microbiologist (pathologist) providing clinical governance (including direct liaison with clinicians and public health officers) is available to discuss unusual and/or unexpected cases;
- In a low prevalence setting, samples with a high cycle threshold (Ct) value should be interpreted with caution; and repeat testing of the same sample and re-sampling considered;

- Ensure close collaboration between the laboratory and local public health units and clinicians to identify potential false positives, ensure clear understanding of test results and potential limitations and, if required, to initiate further testing.

Measuring the performance of SARS-CoV-2 tests

Test performance can be measured by
- analytical sensitivity (the ability of the test to detect a pathogen when it is present)
- analytical specificity (the ability of a test to be negative when a pathogen is not present)
- clinical sensitivity (the percentage of people with infection who will have a positive test)
- clinical specificity (percentage of people without the infection who will have a negative test)

Tests that have lower analytical and clinical specificity, are more likely to lead to false positive results, and these characteristics are relatively fixed. As a result, laboratories generally do not use tests with these characteristics.

The incidence of false positive results is also impacted by the prevalence of the disease in the population undergoing testing. The positive predictive value (percentage of people with a positive test that will have the infection) and negative predictive value (percentage of people with a negative test do not have the infection) are variable, and dependent upon the pre-test likelihood of the person having infection.

For example, if a test with a specificity of 99% is used to test symptomatic passengers on a cruise ship where the likelihood of infection is 50%, the positive predictive value is 99% (i.e. for every 100 people with a positive test result, 1 person will have a false positive result and do not have infection).

However, using the *exact same test*, if a low risk asymptomatic population is tested where the likelihood of infection is 5 in 10,000 (i.e. 0.05%), the positive predictive value is 4.3% (i.e. for every 100 people with a one-off positive test result, 95-96 people will have a false positive result and do not have infection). The use of a confirmatory second assay of equal or greater sensitivity is recommended in these situations.

These examples emphasise the importance of interpreting the test results in light of the pre-test likelihood of infection (risk factors and clinical features), even with a test that has very high specificity.

Table 1: Sources of False Positive PCR Tests, Preventative Measure and Laboratory Responses

Potential Sources of False Positivity	Preventative Measures	Laboratory Responses
1. Laboratory Errors		
Specimen mislabelling – specimen was not collected from the patient identified on the request form or the specimen	• Standard procedures at point of collection for positive patient identification and specimen labelling • Identification of error at data entry/specimen reception prior to testing • Note: if there is complete mislabelling of both the request form and specimen, the error is not identifiable by the laboratory and may only be resolved by re-collection due to investigation of an unexpected result	• Specimen rejected and repeat collection performed
Data entry/specimen receipt transcription errors – specimen is entered under the incorrect patient	• Standard procedures at data entry/specimen reception to minimise errors • Identification of error within the laboratory prior to testing	• Re-check patient details on specimen and repeat testing to determine true result
Contamination of primary specimen with positive specimen	• Standard laboratory procedures to minimise contamination of equipment, solutions and surfaces which may lead to specimen contamination • Use of negative controls to identify contamination • Identification of error at time of analysis and/or reporting due to low level amplification and inconsistent clinical history	• Repeat testing on a newly collected specimen
Contamination of specimen extract with positive specimen extract or positive control material	• Standard laboratory procedures to minimise contamination of equipment, solutions and surfaces which may lead to specimen contamination • Use of negative controls to identify contamination • Identification of error at time of analysis and/or reporting due to low level amplification and inconsistent clinical history	• Repeat extraction and re-testing of original specimen on same and/or different assay • Repeat testing on a newly collected specimen
Data transcription errors – wrong result entered or result entered into wrong patient record	• Standard laboratory procedures to minimise transcription errors • Identification of error may occur at time of reporting	• Re-check results and repeat testing from primary specimen or extracted specimen if necessary
Patient mis-identification following verbal notification of result	• Standard procedures for positive patient identification during verbal notification procedures • Identification by clinician on correlation with formal written/electronic report	• Provision of formal written/electronic report
Misinterpretation of result by the clinician	• Design of laboratory reports to reduce ambiguity and inclusion of interpretive comments to assist in clinician interpretation	
Test result is truly positive but the patient does not have active infection or evidence of disease	• Highly sensitive assays may detect non-viable virus from past infection • Requires clinician assessment of the epidemiology and clinical features	
180 degree rotation of 96 well plates during extraction or assay setup	• Plate markers; second checkers; strategic placement of controls	• Repeat extractions and testing as required
2. Off-target Reactivity		
Amplification of non-specific PCR products	• Assay design • Result analyses and interpretation • Identification at time of analysis and/or reporting due to low level amplification, poor amplification curve and/or inconsistent clinical history	• Repeat extraction and re-testing of original specimen on same and/or different assay and/or genomic sequencing • Repeat testing on a newly collected specimen