A joint venture between The University of Melbourne and The Royal Melbourne Hospital

Updated Report

Post-market validation of a further three serological assays for COVID-19

2 February 2021

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Contents

Post-market validation of a further three serological assays for COVID-19 continued

Executive Summary

Here, we present results of post-market validation of a further three serological assays for the detection of SARS-CoV-2. Previous post-market evaluations were undertaken by the Doherty Institute on a cohort of stored serum prior to the COVID-19 outbreak in Australia, and on samples of serum specimens collected from patients with SARS-CoV-2 infection confirmed by molecular testing. Previous reports were issued in April, June, August, September and October 2020. In September 2020, the Doherty Institute established a collaboration with the National Serology Reference Laboratory, Australia (NRL) to undertake future post-market evaluations using a different set of samples. The new panel of specimens have high volume, are well-characterised and will allow for comparison of performance across test kits. The first NRL report was published in January 2021.

It must be noted that the panel of specimens used in this study have some fundamental differences to those used in previous studies. Therefore, the results of the studies cannot be directly compared. The current NRL panel of specimens are plasma rather than serum. All positive specimens used in the current study were obtained from RT-PCR positive patients with the plasma specimens taken at least 14 days after symptom onset. Previous studies included a subset of specimens obtained within 14 days of onset of symptoms.

Post-market validation of a further three serological assays for COVID-19 continued

Our findings suggest that overall specificity of these three point-of-care tests demonstrate specificity similar to that reported in the manufacturers' claims; however, the sensitivity of the test kits estimated by this study for IgM reactivity was generally lower than that reported by two of the three test kit manufacturers. This could be due to the different set of samples used in this study compared with samples used in the manufacturer's studies.

1. Introduction

This work continues the post-market validation work first reported on 28th April, 2nd June, 10th August, 24th September, 13th October 2020 and 27 January 2021 by the Doherty Institute and the studies conducted by NRL. Following the initial laboratory responses and release of the viral whole genome sequence by Chinese investigators in early January 2020, there was a rapid development of serological assays for COVID-19.[1–3] The first serological tests for COVID-19 were lateral flow immunoassays, also known as serological point-of-care tests (PoCT). The urgent need for diagnostic testing has meant that many test kits have undergone an expedited assessment from the Australian Therapeutic Goods Administration (TGA). As such, post-market validation of COVID-19 diagnostic kits that are listed on the Australian Register of Therapeutic Goods (ARTG) has been undertaken. Here, we present findings from a post-market validation study of a further three serological PoCT (all listed on the ARTG).

2. Methods

2.1 Establishment of patient cohorts and serum samples

Specimens acquired by NRL have been used in this study and will be used in future post market evaluations. The panels were designed to maximise the value of the number of test kits provided for the evaluation (300 test devices; 150 from each of two test kit lots). COVID-19 serology test kits performance was evaluated for the following performance characteristics:

- Clinical Sensitivity
- Clinical Specificity
- Analytical Specificity
- Lot-to-lot Variation

Clinical sensitivity analysis

A total of 100 plasma specimens were obtained from 100 unique patients with SARS-CoV-2 detected by RT-PCR from upper and / or lower respiratory tract specimens. The plasma specimens were collected no less than 14 days post infection. This time-period allows for the development of an immune response. Detectable SARS-CoV-2 IgG was confirmed in each sample using a chemiluminescent immunoassay. Positive specimens were categorised into time-periods from onset of symptoms (Table 1).

Post-market validation of a further three serological assays for COVID-19 continued

Table 1. Number of SARS-CoV-2 positive specimens, collected at different time points after onset of symptoms, included in the post market evaluation study.

Period between onset of symptoms and plasma collection	Week 3 (15-21 days)	Week 4 (22-28 days)	Week 5 (29-35 days)	Week 6 (36 – 42 days)
Number of specimens	8	25	49	18

Clinical specificity analysis

A total of 100 plasma specimens collected from 100 unique blood donations made prior to November 2019 were used to determine the clinical specificity of the assays.

Analytical specificity analysis

Cross-reacting specimens – A total of 25 specimens from individuals that have confirmed past or recent infection with other organisms that may cause cross-reactivity were tested to determine false reactivity. This panel comprised of samples obtained from individuals with evidence of infection with malaria (n=5); Influenza A, Influenza B and CMV IgM positive (n=3 each); acute parvovirus B19 and EBV (n=2 each) and single samples from individuals with acute infections with mycoplasma, parainfluenza, *C. psittaci*, Toxoplasma, rubella and Hepatitis A and B.

Lot-to-lot variation

Two specimens in the clinical sensitivity panel were used to create a doubling-dilution series in negative plasma. The same serial dilutions were tested in both reagent lots to identify lot-to-lot variation.

2.2 Test Kits

A total of three serology PoCTs were included in the current study: Nantong Egens COVID-19 IgM/IgG Rapid Test Kit (Egens), Healgen COVID-19 IgG/IgM Rapid Test Cassette (Healgen), and Newscen COVID-19 IgG/IgM Rapid Test Cassette (Newscen). The test kits were stored in a temperature and humidity-controlled environment. All testing was performed according to the manufacturer's instructions for use (IFU). The same panel of specimens were tested on each test kit.

All test kits assessed in this study are lateral flow serological assay. Common features are that:

i. they are single use immunochromatographic lateral flow tests, for the detection of IgM and/or IgG in serum, plasma or whole blood
ii. the specific SARS-CoV-2 recombinant antigen(s) incorporated into the assay are not described in the IFU

iii. IFUs indicate that test results should not be used as the sole basis for clinical management decisions, requiring interpretation alongside clinical features and other diagnostic (molecular) assays.

Table 2. Test kits, manufacturer and reagent lot numbers tested in post market study.

Test Kit	Manufacturer	Batch Numbers #
Egens	Nantong Egens Biotechnology Co Ltd *Lavinia Medical Pty Ltd*	20200606
		20200402
Healgen	Healgen Scientific Ltd *Southwind International Pty Ltd*	2004173
		2004159
Newscen	Newscen Coast Bio-pharmaceutical Co. Ltd *Kissun Pharmaceuticals Pty Ltd*	Y07G2002
		Y07G2003

Immunochromatographic assays involve the detection of anti-SARS-CoV-2 IgM or IgG antibodies through binding to immobilised recombinant antigen attached to colloidal gold, followed by detection of the conjugates by an anti-human IgM or IgG antibody. A control line is also incorporated, which measures adequacy of fluid flow along the test strip. In general, with respect to the generation of reported performance characteristics limited information was supplied in the manufacturer IFU's regarding:

Post-market validation of a further three serological assays for COVID-19 continued

i. where validation samples were sourced from;
ii. whether plasma, serum, whole blood or a combination of these were used for validation;
iii. what proportion of patients included were confirmed by a result from RT-PCR.

The performance claims specified in the manufacturers' instructions for use are detailed in Table 3.

Table 3: Reported performance characteristics of included serological assays according to manufacturer's instructions for use.

Assay	Sensitivity	Specificity
Egens	IgM = 61.2% [47.3 – 73.6] IgG = 83.7% [71.0 – 91.5] IgG or IgM = Not stated **(n=49)***	IgM = 100% [93.8 – 100] IgG = 100% [93.8 – 100] IgG or IgM = Not stated **(n=58)**
Healgen	IgM = 64.0% [58.1 – 69.5] IgG = 93.4% [89.8 – 95.9] IgG or IgM = 93.8% [90.1 – 96.2] **(n = 289)**	IgM = 99.0% [97.8 – 99.6] IgG = 99.0% [97.8 – 99.6] IgG or IgM = 98.5 [97.1 – 99.3] **(n = 584)**
Newscen	IgM = 72.0% [65.6 – 77.9] IgG = 90.4% [85.7 – 93.9] IgM or IgG =95.8% [93.5 – 98.7] **(n=218)**	IgM = 100% [99.1 - 100] IgG = 99.3% [97.9 – 99.9] IgM or IgG =99.3% [97.9 – 99.9] **(n=419)**

* Samples collected >14 days post infection

2.3 Testing protocol

Testing of the lateral flow assays was performed at NRL by three laboratory technicians, all of whom have undergone previous training in the use of lateral flow assays. Testing was performed exactly as per the IFU. For all testing, lateral flow test strips were each read by two different technicians. A third read by a third technician was undertaken if the first two reads were discordant, with the third read taken as the final result. Reading by all three scorers were made within the time-frame specified by the manufacturer.

Any specimen with an invalid test result was repeated on the same lot number, if sufficient test devices were available. If the subsequent test reported a valid result, that result was used as the final result. If the specimen was repeatedly invalid that specimen was removed from analysis, but the total number of invalid and discordant results were noted.

2.4 Statistical analysis

Clinical sensitivity and specificity – In this study, sensitivity was defined as the reactivity of the assay (IgG only, IgM only and IgG and/or IgM) when testing plasma specimens, taken at least 14 days post onset of symptoms, from patients with SARS-CoV-2 detected by RT-PCR from upper and / or lower respiratory tract specimens. Specificity is defined as the non-reactivity of the assay when testing specimens that do

Post-market validation of a further three serological assays for COVID-19 continued

not contain the analyte (reference results negative). Table 4 details the cross-tab analysis for sensitivity and specificity.

Table 4. Cross-tab analysis of clinical sensitivity and specificity.

		Reference testing results		
Results of assay under evaluation		Specimens from COVID infected individuals	Specimens from individuals not infected with COVID	Total
	Reactive	a (true positives)	b (false positives)	a + b
	Non-reactive	c (false negatives)	d (true negatives)	c + d
	Total	a + c	b + d	a+b+c+d

Sensitivity = $\frac{a}{a+c}$ **Specificity** = $\frac{d}{b+d}$

The exact 95% confidence intervals for binomial proportions are calculated for both sensitivity and specificity.

Lot-to-lot variation – The highest dilution having a reactive test result on Lot 1 was compared with the highest dilution having a reactive test results on Lot 2.

2.5 Ethics

The specimens used in this study were provided to NRL by third-party organisations, including national blood transfusion services. Some specimens with potentially cross-reacting and interfering substances were obtained from commercial organisations. All specimens were collected from individuals with informed consent under various ethics approvals.

3. Results

3.1 Clinical sensitivity analysis

The clinical sensitivity of the anti-SARS-CoV test kits was assessed by comparing the reactivity of the IgG and IgM line against the clinical status of the individual. The results of IgG, IgM and combined IgG and/or IgM sensitivity, post symptom onset is presented in Table 5. There were some differences in the sensitivity and/or specificity reported in this study compared with those reported in the manufacturers' IFU. The sensitivity of IgM reactivity was low compared to claims stated in the IFU for Egens (51.5%) and Newscen (57.6%) but higher than the IFU for Healgen (80.9%). The reported IgG reactivity on positive specimens range from 87.9% for Newscen to 98.9% for Healgen. Sensitivity determined by IgG and/or IgM was higher than each individual antibody class.

Post-market validation of a further three serological assays for COVID-19 continued

Table 5: Comparative performance of serological assays, regardless of day of specimen collection post-symptom onset, for SARS-Cov-2 IgG only, IgM only, and IgG and/or IgM detection**.**

Performance Characteristic / Test Assay	Sensitivity (%) [95% CI]	Specificity (%) [95% CI]
Egens IgM	51.5 [41.3 – 61.6]	95.0 [88.2 – 98.1]
Egens IgG	94.9 [88.1 – 98.1]	92.0 [84.4 – 96.2]
Egens IgM or IgG	**97.0 [90.8 – 99.2]**	**91.0 [83.2 – 95.5]**
Healgen IgM	80.9 [71.2 – 88.0]	91.0 [83.2 – 95.5]
Healgen IgG	98.9 [93.4 – 99.9]	97.0 [90.8 - 99.2]
Healgen IgM or IgG	**100 [95.1 - 99.9]**	**91.0 [83.2 – 95.5]**
Newscen IgM	57.6 [47.2 – 67.3]	97.0 [90.8 – 99.2]
Newscen IgG	87.9 [79.4 – 93.3]	98.0 [92.3 – 99.7]
Newscen IgM or IgG	**90.9 [83.0 – 95.5]**	**96.0 [89.5 – 98.7]**

A detailed analysis of sensitivity of IgM only, IgG only and IgG and/or IgG reactivity on positive samples categorised by period of time post onset of symptoms is presented in Tables 6 – 8 below.

Post-market validation of a further three serological assays for COVID-19 continued

Table 6: Comparison of the Egens IgM/IgG Antibody to Coronavirus (SARS-CoV-2) reactivity with 100 patients with confirmed COVID-19 infection, stratified by days post-symptom onset.

Days post-symptom onset	Samples (n)	IgM detected (%) [95% CI]	IgG detected (%) [95% CI]	IgM or IgG (%) [95% CI]
15-21	8	2 25.0% [4.5 - 64.4]	7 87.5% [46.7 - 99.3]	7 87.5% [46.7 - 99.3]
22-28	25	13 52.0% [31.8 – 71.7]	22 88.0% [67.7 - 96.8]	23 92.0% [72.5 - 98.6]
29-35	49	22 45.8% [31.6 – 60.7]	47 97.9% [87.5 – 99.9]	48 100.0% [90.8 - 99.8]
36-42	18	14 77.8% [51.9 – 92.6]	18 100.0% [78.1 - 99.5]	18 100.0% [78.1 - 99.5]
Total	**100**	**51** **51.5% [41.3 - 61.6]**	**94** **94.9% [88.1 – 98.1]**	**96** **97.0% [90.8 – 99.2]**

Table 7: Comparison of the Healgen IgM/IgG Antibody to Coronavirus (SARS-CoV-2) reactivity with 100 patients with confirmed COVID-19 infection, stratified by days post-symptom onset.

Days post-symptom onset	Samples (n)	IgM detected (%) [95% CI]	IgG detected (%) [95% CI]	IgM or IgG (%) [95% CI]
15-21	8	3 37.5% [10.2 – 74.1]	7 87.5% [46.7 - 99.3]	8 100.0% [59.8 - 98.8]
22-28	25	21 84.0% [63.1 – 94.7]	25 100.0% [83.4 - 99.6]	25 100.0% [83.4 - 99.6]
29-35	49	37 84.1% [69.3 – 92.8]	44 100% [90.0 – 99.8]	44 100% [90.0 – 99.8]
36-42	18	15 88.2% [62.3 – 97.9]	17 100% [77.1 - 99.5]	17 100% [77.1 - 99.5]
Total	**100**	**76** **80.9% [71.2 - 88.0]**	**93** **98.9% [93.4 – 99.9]**	**94** **100% [95.1 – 99.9]**

Post-market validation of a further three serological assays for COVID-19 continued

Table 8: Comparison of the Newscen IgM/IgG Antibody to Coronavirus (SARS-CoV-2) reactivity with 100 patients with confirmed COVID-19 infection, stratified by days post-symptom onset.

Days post-symptom onset	Samples (n)	IgM detected (%) [95% CI]	IgG detected (%) [95% CI]	IgM or IgG (%) [95% CI]
15-21	8	3 37.5% [10.2 – 74.1]	6 75.0% [35.6 - 95.5]	7 87.5% [46.7 - 99.3]
22-28	25	16 64.0% [42.6 – 81.3]	23 92.0% [72.5 - 98.6]	23 92.0% [72.5 - 98.6]
29-35	49	27 55.1% [40.3 – 69.1]	42 85.7% [72.1 – 93.6]	43 87.8% [74.5 – 94.9]
36-42	18	11 64.7% [36.6 - 84.7]	16 94.1% [69.2 – 99.7]	17 100% [77.1 – 99.5]
Total	**100**	**57** **57.6% [47.2- 67.3]**	**87** **87.9% [79.4 – 93.3]**	**90** **90.9% [83.0 – 95.5]**

3.2 Clinical specificity analysis

The results of the specificity analysis for anti-SARS-Cov-2 test kits is presented in Table 5. All test kits had an estimated specificity of 92.0% or greater for IgG only. Specificity for anti-SARS-CoV-2 IgM ranged from 91.0% to 97.0% (Table 5).

3.4 Analytical Specificity

All three test kits reported false positive results for one or more potentially cross-reactive samples. Egens reported a IgG false reactive result for one influenza A and two antinuclear factor positive samples and IgM false positive results for one CMV IgM reactive and four rheumatoid factor positive samples. The Healgen assay

reported a false positive IgM for an influenza B positive sample and reported invalid results for two anti-DNA positive samples; an icteric and a lipaemic sample. Newscen reported false positive IgM results for a CMV IgM positive sample and three rheumatoid positive samples, and reported false positive IgG results for two antinuclear factor positive samples.

3.5 Lot to lot analysis

In general, the IgG and IgM results from testing the dilution series in both reagent lots demonstrated equivalent reactivity (Table 11).

Post-market validation of a further three serological assays for COVID-19 continued

Table 11. Results of testing dilution series of two positive specimens to determine lot to lot comparison of the COVID-19 IgG/IgM Rapid Tests. The highest dilution recording a positive test result was determined as the end point.

Assay [Lot number]	Test Results Sample 1		Test Results Sample 2	
	IgG	IgM	IgG	IgM
Egens 20200606	1:32	1:32	1:16*	1:4
Egens 20200402	1:32	1:16	1:8	1:4
Healgen 2004173	1:128	1:32#	1:8	1:2
Healgen 2004159	1:64	1:32	1:8	1:4
Newscen Y07G2002	1:16	1:16	1:4	Neg
Newscen Y07G2003	1:16	1:8	1:8	Neg

* IgG and IgM reactivity was detected at 1:128
# IgM reactivity was detected at 1:128
Neg – No reactivity detected

Post-market validation of a further three serological assays for COVID-19 continued

4. Discussion

Results of post-market evaluation of a further three COVID-19 serology rapid test are presented. This study was performed by NRL, using a different panel of specimens than the previous Doherty reports. As with previous reports, the results of each test kit evaluated were within the stated IFU range for specificity. The sensitivity estimated by this study for IgM reactivity was lower than that reported by two manufacturers. Note that manufacturers make varying sensitivity claims for IgG only, IgM only and combined IgG/IgM and use different criteria to establish the reference result.

For detection of anti-SARS-CoV-2 IgG and/or IgM, the sensitivity of the three test kits ranged from 90.9% for Newscen to 100% for Healgen. Specificity for IgG/IgM ranged from 91.0% for Healgen and Egens; to 96.0% for Newscen.

There was no significant lot-to-lot variation detected for any test kit for IgG or IgM reactivity.

5. Acknowledgements

We thank the NRL scientific and technical staff who compiled the panel of specimens and performed the testing.

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