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**Highlights**

- Haemaglutination assay (HAT) presence of antibodies to SARS-CoV-2
- It detects antibodies that correlate with neutralizing activity.
- HAT assay is a very cheap assay, with high sensitivity and specificity.
Comparison of two assays to detect IgG antibodies to the receptor binding domain of the SARS-CoV-2 as a surrogate marker for assessing neutralizing antibodies in COVID-19 patients

Running title: Surrogate assays for SARS-CoV-2 neutralizing antibodies

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ABSTRACT

Background: Neutralizing antibodies (NAbs) are important for protection against COVID-19 re-infection. We compared two assays that are correlated with NAbs, the haemagglutination test (HAT) and surrogate virus neutralization test (sVNT).

Methods: The specificity of the HAT was compared with the sVNT and the sensitivity and persistence of antibodies in patients with varying severity of illness was assessed in cohort of 71 patients at 4 to 6 weeks and 13-16 weeks. The kinetics was assessed in first, second and third week in patients with varying severity of acute illness.

Results: The specificity of the HAT was >99%, and sensitivity was similar to lower than the sVNT. The levels of HAT significantly and positively correlated (Spearman’s r= 0.78, p<0.0001) with those of the sVNT. Patients with moderate and severe illness had higher in the HAT titres compared to those with mild illness. 6/7 patients with severe illness had a titre of >1:640 during the second week of illness, whereas only 5/31 patients with mild illness had a titre of >1:160 in the second week of illness.

Conclusions: Since the HAT is a simple, and very cheap assay to perform, it would be ideal to use as an indicator of NAbs in resource-poor settings.

KEY WORDS COVID-19, disease severity, haemagglutination assay, surrogate neutralization assay, neutralizing antibodies
BACKGROUND

There are many antibody assays currently in use to determine IgG, IgM and IgA specific to the SARS-CoV-2 (Algaissi et al., 2020, Sun et al., 2020, Vogelzang et al., 2020). While some of these assays measure total antibodies to mainly the spike protein receptor binding domain (RBD), some measure IgM or IgG responses to S1, S2 or the nucleocapsid protein (Sun et al., 2020, Vogelzang et al., 2020). While these assays are sometimes used in conjunction with the PCR assays, some are used to detect those who have had asymptomatic infection and in sero-surveillance studies (Galipeau et al., 2020). Even though the RT-PCR is the gold standard in identifying those who are acutely infected with SARS-CoV-2, serological tests can contribute by providing more accurate estimates of past SARS-CoV-2 infections and immune status of the population (Ghaffari et al., 2020). However, although different antibody assays can be used to identify those who have been exposed to the virus, only assays that measure, or correlate with, neutralizing antibodies (NAb s), are likely to provide evidence of antibodies that are more likely to protect individuals against re-infection (Galipeau et al., 2020).

NAb s against the SARS-CoV-2 are mainly produced against the RBD of the viral spike protein (Kreer et al., 2020, Ni et al., 2020). The gold standard for determining NAb is plaque reduction neutralization test (PRNT) which requires BSL-3 facilities and is time consuming (Galipeau et al., 2020). With COVID-19 spreading at an exponential rate around the world, many assays have been developed to measure NAb that can be carried out within a few hours in a BSL-2 facility (Tan et al., 2020, Townsend et al., 2020). One such assay is the surrogate virus
neutralization test (sVNT) which measures the percentage of inhibition of binding of the RBD of
the spike protein to recombinant ACE2 (Tan et al., 2020). Further, Townsend and colleagues
have developed an haemagglutination test (HAT) to measure antibodies to the RBD where the
RBD of the virus is linked to a nanobody IH4, specific for a conserved epitope within
glycophorin A on red blood cells (RBCs) (Townsend et al., 2020). In the presence of antibodies
to RBD, IH4-RBD-6H bound RBC will show agglutination (Townsend et al., 2020). Since the
majority of neutralising antibodies are directed at the RBD (Kreer et al., 2020, Ni et al., 2020),
and the level of antibodies detected by the HAT assay correlate with neutralising IC50
(manuscript in preparation), this assay can be used as an inexpensive test to predict NAb in
research and community settings where high throughput assays are required (Townsend et al.,
2020). Therefore, in order to determine the usefulness of the HAT, we proceeded to compare the
performance of the HAT with an existing assay, which is used as a surrogate assay to measure
the presence of Nabs by blockade of ACE2 binding by the RBD.
METHODS

Haemagglutination test (HAT) to detect NAbs

The HAT was carried out as previously described (Townsend et al., 2020). Briefly, red blood cells from an O negative donor were mixed with the IH4-RBD-6H (a nanobody against a conserved glycophorin A epitope on red cells, linked to the RBD of SARS-CoV2) and incubated for one hour with serum. Phosphate buffered saline was used as a negative control. At the end of the incubation the plate was tilted for 20 seconds and then photographed. The photograph of the plate was read by two independent readers to examine the “teardrop” formation indicative of a negative result. A complete absence of “teardrop” formation was scored as positive and any flow of “teardrop” was scored as negative.

The HAT titration was performed using 11 doubling dilutions of serum from 1:20 to 1:20480, to determine the NAb titre. The NAb titre for the serum sample was defined by the last well in which the complete absence of “teardrop” formation was observed.

Surrogate neutralizing antibody test (sVNT) to detect NAbs

The surrogate virus neutralization test (sVNT) (Tan et al., 2020), which measures the percentage of inhibition of binding of the RBD of the S protein to recombinant ACE2 (Tan et al., 2020) (Genscript Biotech, USA) was carried out according the manufacturer’s instructions. Inhibition percentage ≥ 25% in a sample was considered as positive for NAbs.
Patients

Patients confirmed of SARS-CoV2 infection based on the positive RT-PCR were recruited from the National Institute of Infectious Diseases (NIID), Sri Lanka. They were followed throughout their illness while they were in hospital and the severity grading was based on the worst severity while in hospital. Clinical disease severity was classified as mild, moderate and severe according to the WHO guidance of COVID-19 disease severity (WHO, 2020). For this study we recruited two cohorts of patients. Serum samples from the patient cohort 1 (n=50) was used to determine the correlation of HAT tires with sVNT levels and longitudinal changes in SARS-CoV-2 Nabs levels. From the first cohort of patients, blood samples were obtained, during the first and second week and again when they were discharged from hospital (4 to 6 weeks since onset illness) (table 1). The duration of illness was defined from the day or onset of symptoms and not the day of PCR positivity or admission to hospital. The duration of illness was defined as the day of onset of symptoms and not day of PCR positivity or admission to hospital. 21 patients showed PCR positivity for more than 25 days since onset of symptoms and we categorized them as prolonged shredders. All the prolonged shredders were either asymptomatic or had mild COVID-19. Based on the WHO COVID-19 disease classification, six patients had severe illness, five moderate illness, 10 mild illness, and 21 mild illness but with prolonged viral shedding for >25 days(WHO, 2020).
Serum samples from the patient cohort 2 (n=66) were used to study the sensitivity of HAT compared to the sVNT and to determine the persistence of antibody levels in these patients. Details of both these patient cohorts and sampling time points are given in table 1. There were 24 prolonged shredders in this patient cohort and they were either asymptomatic or had mild COVID-19.

Ethical approval was received by the Ethics Review Committee of Faculty of Medical Sciences, University of Sri Jayewardenepura. Informed written consent was obtained from patients.

**Statistical analysis**

The correlation of HAT titres with percentage inhibition of values from the sVNT was assessed with Spearman’s correlation by GraphPad Prism 8 version 8.4.2. The sensitivity of the HAT and sVNT in detecting SARS-CoV-2 antibodies at 4 to 6 weeks and 13 to 16 weeks following infection and was assessed by using the chi-square test.
RESULTS

Determining Specificity of HAT to measure antibodies against SARS-CoV-2

As some antibody assays for SARS-CoV2 are known to detect cross-reactive antibodies to other seasonal coronaviruses (Galipeau et al., 2020), we initially determined the specificity of HAT in the Sri Lankan population (n=110) from samples collected from febrile patients during the years 2017 and 2018. The HAT was performed on serum diluted at 1:20. All these samples were from patients with confirmed dengue viral infection based on RT-PCR and none of the patients had any respiratory symptoms. Except one sample all the serum samples tested negative. Therefore, the specificity of this assay was found to be >99% as previously described (Townsend et al., 2020). The serum sample that tested positive with HAT was from a patient who had an acute infection due to the dengue virus serotype 3.

Correlation of HAT with sVNT in determining the SARS-CoV-2 Nabs levels

In order to determine the performance of the HAT in comparison to the sVNT in patients with acute illness (patient cohort 1), we compared the HAT titres with values given by the sVNT. The HAT titre of the sample was determined by the last well in which the complete absence of “teardrop” formation was observed (Figure 1A). We found the titres of the HAT significantly and positively correlated (Spearman’s r= 0.78, p<0.0001) with levels of ACE2 blocking antibodies obtained from sVNT (Figure 1B). All the samples which were negative for HAT,
were also negative by sVNT. However, in 6 individuals the HAT was positive (titre >1:20), while sVNT was negative during the first week of illness.

Longitudinal changes of antibodies in COVID-19 detected with HAT

We then proceeded to study the longitudinal changes in the Ab titres from patients (patient cohort 1) with varying severity of clinical disease. Patients with moderate and severe illness had higher and persistence NAb levels (higher titres in the HAT) (Figure 2A) compared to those with mild illness with and without prolong shedding (Figure 2B). Except for one patient with severe illness who had a titre of >1:160, all other patients with severe illness had a titre of >1:640 (6/7) during the second week of illness. In contrast, only 5/31 patients with mild illness had a titre of >1:160 in the second week of illness. A similar pattern of antibody kinetics was observed previously in patients with varying severity of illness using the sVNT assay (Jeewandara C. et al., 2021).

The sensitivity of the HAT with sVNT in patients with COVID-19

In order to compare the sensitivity of HAT with sVNT, we proceeded to compare these two assays in samples obtained at 4-6 weeks (n=66) since onset of illness and samples obtained between 13-16 weeks (n=66) since onset of illness (patient cohort 2). Of these 66 patients, 2 had severe illness, 3 moderate illness, 9 mild illness and 52 had completely asymptomatic illness. HAT was carried out at 1:20 dilution and complete absence of “teardrop” formation was scored as positive result and any flow of “teardrop” was scored as negative result. Between 4 to 6 weeks of illness 49/66 (74.2%) of patients gave a positive result for HAT while 48/66 (72.7%) gave a
positive result with the sVNT. Therefore, at 4 to 6 weeks, the sensitivity of the HAT was comparable to the sVNT. At time point B 37/66 (56%) gave a positive result for HAT, while 33/66 (50%) gave a positive result with the sVNT. All the samples which were negative for HAT, were also negative by sVNT. Therefore, while the specificity of HAT was similar to that of the sVNT, the sensitivity appeared to slightly higher (56% vs 50%).

All those with moderate or severe (n=5) illness had NAb detected by HAT during 4 to 6 weeks of illness, and by 13-16 weeks of illness. 24 of those with mild/asymptomatic illness had prolonged shedding (PCR positivity for >25 days). During 4-6 weeks of illness, 16/24 (66.6%) prolonged shedders were positive by HAT and by sVNT (the same 16 individuals were positive by both). Further 33/42 (79%) of those who cleared the virus early were positive by HAT and 32/42 (76%) by sVNT. By 13 to 16 weeks of illness only 12/24 (50%) of the prolonged shedder were positive by HAT and 9/24 (37.5%) by sVNT compared to 24/42 (57%) 11/40 (27.5%) who cleared the virus early being positive by HAT and 23/42 (55%) 23/40 (57.5%) by sVNT. However, these differences were not statistically significant.

These are the first results to measure the HAT in asymptomatic patients over such a prolonged period. It will be important to follow these individuals longitudinally to see whether asymptomatic infection is fully protective against reinfection in this cohort, and whether the HAT result has any utility in predicting susceptibility.
DISCUSSION

In this study we have compared two assays for assessing the presence of Abs against the SARS-CoV2, each with a correlation with neutralising titres, which can be used in a BSL-2 laboratory. The HAT was found to correlate well with the Abs levels given by the sVNT and was found to have a comparable sensitivity during acute illness and up to 13 to 16 weeks since onset of illness. The assay was also found to have a high specificity (>99%) in the samples which were assessed before any individuals in Sri Lanka would have been exposed to SARS-CoV-2. However, due to the non-availability of a BSL-3 laboratory within the country, it was not possible to compare the HAT assay in Sri Lankan patients and therefore, the assay was compared with the sVNT, which evaluated ACE2 blocking antibodies and has shown to be correlate well with neutralizing antibodies(Tan et al., 2020). The titres given by the HAT assay were higher during early infection in those with severe and moderate illness compared to those with mild illness as described previously (Jeewandara C. et al., 2021, Lynch et al., 2021). Although the reasons for detection of higher levels of Abs during early infection is not clear, it could be due increased production by extrafollicular B cells (Galipeau et al., 2020, Lee et al., 2020, Woodruff et al., 2020), or the more efficient ability of IgM to cross-link red cells.

In Sri Lanka, until early July 2020, all individuals with COVID-19 were discharged from treatment centres/hospitals after two PCRs, done 24 hours apart became negative. Therefore, repeated PCRs we carried out on hospitalized patients to enable to be discharged once, two
consecutive PCRs are negative. This enabled us to detect those who had prolonged shedding of the virus for 4 to 9 weeks since onset of illness. Interestingly, these prolonged shedders were less likely to have adequate levels of NAbs at 4-6 weeks and 13-16 weeks compared to those who became PCR negative early, although this was not statistically significant. It is possible that these individuals continued shedding PCR detectable viral material, for many weeks due to reduced production of NAbs or T cells, which should be further investigated.

Although cross-reactive antibodies specific to other coronaviruses are known to interfere with the specificity of SARS-CoV2 serological assays (Galipeau et al., 2020), the HAT showed high specificity (99%) and therefore, it is unlikely to pick up such cross-reactive antibodies. In addition, it was found to have an equal sensitivity to the sVNT in detecting RBD-specific antibodies when compared to the sVNT during 4-6 weeks and 13-16 weeks from onset of illness, and in asymptomatic cases. These are the first results with the HAT assay applied to asymptomatic infections, and clearly show a reduced sensitivity ~50 73% compared to about one month after symptomatic infections (~90%) (Townsend et al., 2020). In other work (manuscript in preparation) we have shown a strong correlation between the HAT titre and authentic virus neutralisation titre (IC50.) Prospective measurements in adequate cohort studies will be needed to tease apart the relationship between these measurements and protection from re-infection. In addition, with the worldwide vaccination drive against COVID-19, the HAT can be very useful especially in resource poor settings to measure seroconversion rates and antibody levels after vaccination. We have successfully used the HAT to measure the antibodies titres against the RBD of SARS-CoV2 one month after AZD1222/Covishield vaccine in Sri Lanka (Jeewandara Chandima et al., 2021).
In summary, the HAT assay is a very cheap assay, with high sensitivity and specificity, which is a surrogate test to detect the presence of Abs to SARS-CoV-2 that correlate with neutralising activity. As it does not require any specific equipment, it would be a valuable and very cheap tool, in resource-poor settings. As it appears to have comparable sensitivity to the sVNT assay, it might be less suitable for population screening to identify previously exposed individuals.

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**Declaration of conflicts of interests**

None.
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Figure legends

**Figure 1.** Correlation of antibody levels determined by HAT titration and sVNT.

A: An example of HAT titration carried out for a sample. The HAT titration was carried out using 11 doubling dilutions of the serum from 1:20 to 1:20480 (the titres are indicated as 20, instead of 1:20). HAT titre of the sample was determined by the last well in which the complete absence of “teardrop” formation was observed (marked with a black solid-line circle), indicating a titre if 1:40 in this patient.

B: Correlation of antibody levels determined by HAT titres and the percentage inhibition of binding of the RBD to recombinant ACE2 by the serum sample by the sVNT assay, Spearman's r=0.79, p<0.0001.

**Figure 2.** Antibody levels were determined by HAT titration with the varying severity of COVID-19. The HAT titration was carried out using 11 doubling dilutions of the serum from 1:20 to 1:20480 (the titres are indicated as 20, instead of 1:20) in patient’s serum obtained during the first week, second week and 4 to 6 weeks since onset illness. A: Longitudinal changes of HAT titres with the duration of the illness in patients with severe illness (n=7) and moderate illness (n=5) B: Longitudinal changes of HAT titres with the duration of the illness in patients with mild prolonged shedders (n=21) and mild illness without prolong shedding (n=10)
Table legend

Table 1. The disease severity information and bleeding time points for the two patient cohorts used in the study

|                          | Patient cohort 1 | Patient cohort 2 |
|--------------------------|------------------|------------------|
|                          | N=50             | N=66             |
| Mean Age                 | 42               | 43               |
| Gender: Male/Female      | 37/13            | 32/34            |
| Time points for bleeding since the onset of symptoms |                   |                   |
|                          | First week       | 4-6 weeks        |
|                          | Second week      | 13-16 weeks      |
|                          | 4-6 weeks        |                  |
| Asymptomatic COVID-19    | 8                | 52               |
| Mild COVID-19            | 31               | 9                |
| Moderate COVID-19        | 5                | 3                |
| Severe COVID-19          | 6                | 2                |
Fig. 1
Fig. 2