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Research article

Validation of a focus reduction neutralization test (FRNT) to rapidly titrate human SARS-CoV-2 neutralizing antibodies by using the CTL Immunospot S6 universal analyzer

Ilaria Puglia [a,b,*], Marialuigia Caporale [a,b], Claudia Casaccia [b], Fabrizia Valleriani [b], Romolo Salini [b], Shadia Berjaoui [b], Cristina Marfoglia [b], Lucilla Ricci [b], Alessio Lorusso [b]

[a] PhD National Programme in One Health Approaches to Infectious Diseases and Life Science Research, Department of Public Health, Experimental and Forensic Medicine, University of Pavia, Pavia, 27100, Italy
[b] Istituto Zooprofilattico Sperimentale dell' Abruzzo e Molise, Teramo, Italy

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ABSTRACT

In this paper we describe the validation of a focus reduction neutralization test (FRNT) to quantitate human SARS-CoV-2 neutralizing antibodies by using the CTL Immunospot S6 Universal Analyzer. We employed a previously published protocol and compared its performances to a well-established and traditional serum-neutralization assay (SN). To assess diagnostic sensitivity, a total number of 201 human sera positive by SN for SARS-CoV-2 NAbs were processed: 196/201 tested positive by FRNT50 (97.51 %). A diagnostic specificity of 100 % was obtained by evaluating 206 negative serum samples. Repeatability of the test was evaluated by determining the intra and inter-assay coefficient of variation (CV). A standard deviation of 0.83 and a CV of 13 % were evidenced demonstrating an acceptable reproducibility of the assay. Moreover, a Cohen's Kappa of 0.975 was obtained proving an extremely high level of agreement between the FRNT protocol and the SN. Despite an acceptable correlation between methods ($p < 0.05$), FRNT demonstrated a statistically significant increase in NAbs titres compared to SN as well as higher data variability and asymmetry. These discrepancies could be attributed to FRNT sensitivity or most probably to the subjective interpretation of SN, although this aspect needs to be further investigated with a more representative number of samples.

Basing on our results, it is reasonable to replace the SN with the FRNT assay as, with this, fast processing time (less than 2 days) and operator bias-free results registrations are guaranteed.

1. Introduction

Neutralizing antibodies (NAbs) are proxy indicators of protection against pathogens, providing critical information about the immune response [1–5]. Moreover, assessing the presence and persistence of antibodies is essential to evaluate the efficacy of vaccines and to design vaccination and/or revaccination programmes [6,7]. Following the onset of COVID-19 pandemic, several *in vitro* assays have been established to monitor, in association with molecular assays, the viral spread and the antibody-mediated immune response.

* Corresponding author. Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise; Italy.
E-mail address: i.puglia@izs.it (I. Puglia).

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Several methods based on the serum-neutralization (SN) test were developed to detect and quantify SARS-CoV-2 neutralizing antibodies in human serum samples [1,7–9].

Currently, the SN is considered the gold standard to investigate the presence of NAbs binding to the S protein in both naturally infected and vaccinated individuals [10]. Briefly, SN consists in the evaluation of the virus-induced cytopathic effect (CPE) inhibition on cell-culture after the inoculation of serum/virus mixtures. However, this technique requires specialized personnel, it is time-consuming taking up to four/five days [11,9] with a significant operator-dependent bias in the reading steps. Furthermore, it is difficult to automate because the operator needs to microscopically assess the visible CPE and to analyze data by hand or by using spreadsheets calculations to determine neutralization titres [12].

The focus reduction neutralization tests (FRNT) could be considered as a valid alternative to traditional SN. This method determines the percentage of reduction of virus infectivity quantifying immunostained foci in cell monolayers by using a computerized system. Thus, FRNT enables a substantial increase in assay throughput and standardization, by-passing the subjective interpretation of SN [12].

In this study we describe the validation of a FRNT protocol previously described [1] employed for a fast SARS-CoV-2 NAbs titration out human sera.

2. Materials and methods

Ethical Approval. No ethical approval was specifically requested since human serum samples analysed in this study derived from the official monitoring activities for SARS-CoV-2 antibodies performed by the Local Public Health Authority of Abruzzo Region (Prot: 2020/0007891/GEN/GEN). Each participant provided written informed consent to participate in the study and for their data to be published according to the Artt. 7 e 13 of Regolamento EU 2016/679.

Samples and validation process. A total of 407 human serum samples were enrolled to adapt and validate, to our settings, the protocol from Padoan [1]. More in detail, 201 positive sera for SARS-CoV-2 NAbs by means of the standard SN assay employed at IZSAM [11] were included to assess diagnostic sensitivity. These samples were randomly selected from a cohort of SARS-CoV-2 infected individuals between 2020 and 2022, regardless of the disease severity. In addition, 206 SN-negative samples for SARS-CoV-2 NAbs, from unvaccinated and uninfected subjects, were enrolled to assess diagnostic specificity. Each sample was obtained from a different individual. Repeatability and reproducibility of FRNT were evaluated by determining the intra and inter-assay coefficient of variation (CV). As references, two control serum samples routinely used in SN at IZSAM were employed. To assess repeatability, these serum samples were used undiluted and analysed in 40 replicates in two different runs carried out by the same operator in different days. Furthermore, to ensure reproducibility, reference sera were both analysed 20 more times by a different operator using a separate batch of reagents. From the two before-mentioned sets of data the mean value, the standard deviation (SD) and the coefficient of variation (CV) were calculated.

Statistical analysis. The diagnostic sensitivity and specificity were calculated by using the MedCalc Software (Version 22.017). The 95 % confidence intervals (CI) were calculated using the Clopper-Pearson confidence intervals or the logit confidence intervals given by Mercaldo [13]. To investigate the qualitative agreement between FRNT and SN, the Cohen's Kappa was calculated as described by McHugh [14]. Instead, for a quantitative comparison, the Wilcoxon-Mann-Whitney was performed by using the XLSTAT 2022.5.1.1390 (Statistical Software for Excel).

Details regarding the cell lines, viruses, and the complete protocol for the validation process can be found in the Supplementary Material.

3. Results

The SARS-CoV-2 strain 2021TE288699 (Delta variant, lineage B.1.617.2 by Pangolin COVID-19 Lineage assigner) stock, employed for SN and FRNT, had a titre of $10^{6.68}$ $TCID_{50}$/ml (end-point titration), and $10^{6.3}$ FFU/ml (FRNT50) by using the CTL Immunospot S6 Universal Analyzer. A total of 407 serum samples tested by the traditional SN were analysed by FRNT. Out of 201 positive serum enrolled to assess diagnostic sensitivity, 196 tested positive by FRNT50 thus having a sensitivity of 97.51 % (95 % CI: 94.29–99.19 %). As far as specificity is concerned, all negative sera (206) were confirmed as negative determining a diagnostic specificity of 100 % (95 % CI: 98.23 %–100 %) (Table 1).

Repeatability and reproducibility of FRNT were evaluated by testing two reference sera (2021TE2571/1 and 2020TE90363/7). Both sera were tested in 60 replicates carried out by two different operators and conditions. Expected results were confirmed in all 60

Table 1
Data from validation of the FRNT protocol. %, percentage; 95 % CI, 95 % confidence interval.

Parameter	Data from FRNT
Diagnostic sensitivity % (95 % CI)	97.51 (94.32–98.90)
Diagnostic specificity % (95 % CI)	100 (98.56–100)
Repeatability % (95 % CI)	13 %
Reproducibility % (95 % CI)	13 %
K di Cohen % (95 % CI)	0.975 (0.96–0.99)

replicates, thus 100 % of qualitative repeatability and reproducibility were obtained (95 % CI: 92.95 %–100 %). As for the quantitative determination of the positive reference serum sample, of which in SN was 160 (log_2 7.32), a mean value of 6.40 log_2 FRNT50 was obtained. In this case a SD of 0.83 and a 13 % CV were demonstrated, thus giving an acceptable reproducibility of the assay (Table 1).

The Cohen's Kappa, used to investigate the qualitative agreement between the FRNT and the SN, was determined. By considering the 407 serum samples (201 positives and 206 negatives), a Kappa of 0.975 (95 % CI: 0.96–0.99) was calculated, therefore proving an extremely high level of agreement between the two methods.

Finally, for a quantitative comparison between methods, the Wilcoxon test was performed. In Table 2, a summary statistics from positive results is reported. FRNT50 and SN generated a mean of 6.05 and 5.58 log_2, respectively; FRNT50 reported a higher standard deviation with respect to that of SN (SD: 2.57 FRNT50; 1.94 SN). Considering the classical significance level (α 0,05), an approximation has been used to compute the p-value (*P*). It was lower than α, thus the alternative hypothesis, for which SN and FRNT distributions are statistically different, was accepted (Fig. 1). Specifically, the FRNT method demonstrated a statistically significant increase in NAbs titres compared to those of SN (Fig. 1). Additionally, the boxplot shows significant discrepancies in data variability and higher asymmetry, which are greater in the case of FRNT50. However, the correlation with SN was acceptable considering the calculated p-value ($P < 0.05$). These data are confirmed by the descriptive statistical graphs in Fig. 2. FRNT50 titres are generally higher compared to those from SN (positive values in the graph), while only a lower number of sera showed higher titres by SN (negative values in the graph) (Fig. 2a). Two extreme cases can be observed at the highest positive and negative values, respectively. In the first case (difference of log_2 of 6), a serum sample with a titre of 20 by SN exhibited a FRNT50 titre of 1280. In the second case (difference of log_2 equal to −6), a sample tested negative in FRNT50 had a SN titre of 40. Same results are expressed as quartiles in the Q-Q plot of Fig. 2b.

4. Discussion and conclusion

In this work, we validated a focus reduction neutralization test (FRNT), starting from a previously published procedure, to titrate SARS-CoV-2 neutralizing antibodies out of human sera [1]. More specifically, at that time the authors have compared their protocol with different methods, such as ELISA kits and several chemiluminescence immunoassays. They also evaluated the analytical and clinical performances of the mentioned methods as well as the correlation with serum samples neutralizing activity [1,15–17,18].

The aim of the present study was to compare FRNT and the SN test. By considering FRNT50 as outcome, a diagnostic specificity of 100 % was obtained. Instead, a 97.51 % diagnostic sensibility was recorded as five positive serum samples tested positive in SN (all samples had a titre of 10) but negative in FRNT. The FRNT method demonstrated a statistically significant increase in NAbs titres compared to those of SN as well as higher data variability and asymmetry. In any case, correlation between methods was acceptable ($p < 0.05$). The discrepancies are likely due to the higher FRNT sensitivity or, in the opposite scenario, to the operator-biased interpretation of results of the SN. Nevertheless, this aspect needs to be further investigated with a more representative number of samples.

However, the computed Cohen's Kappa value of 0.975 attested to an exceptional agreement between the two methods.

SN requires at least 4–5 days of incubation to observe CPE and it is significantly related to subjectivity-bias since operator needs to microscopically evaluate the CPE and to analyze data manually. This factor represents an obstacle for assay validation, which may lead to difficulties in using data as part of a regulatory submission [9]. At the opposite, with this validated FRNT, plates are fixed after 24 h incubation reducing the turnaround time of the SN considerably. In this way, the antibody titre of a given sample is determined in 30/32 h. Additionally, after FRNT immune-staining, plates are automatically scanned, and virus-induced foci directly counted by the CTL Immunospot S6 Universal Analyzer (Immunospot, Cleveland, USA). The use of this computerized counting system significantly increases automation, thus standardization by reducing the error rate in the interpretation of results. Subsequently, accuracy and throughput improve.

This work has reasonably some flaws. Firstly, a limited number of serum samples was enrolled. Therefore, human sera were retrospectively and randomly chosen within those available at IZSAM. Consequently, specimen selection as well as the lack of clinical data introduce *per se* biases which may impact the neutralizing antibodies concentration. Additionally, FRNT cross-reactivity with related viruses was not evaluated. Anyway, to reduce this bias a high SARS-CoV-2 specific primary monoclonal antibody was selected; this antibody was characterized, according to the manufacturers, by the absence of cross-reactivity in ELISA with MERS-CoV, and low pathogenic human-CoVs (229E, NL63, HKU1, and OC43).

In conclusion, this paper describes the validation process of the FRNT protocol. The findings suggest that it could be considered as a viable alternative to the traditional serum neutralization test.

Data availability statement

The data that support the findings of this study have not been deposited into a publicly available repository but will be made available on request from the corresponding author [IP].

Table 2
Summary statistics from Wilcoxon test. Obs, observations; Min, minimum; Max, maximum; SD, standard deviation.

Variable	Observations	Obs. with missing data	Obs. without missing data	Min.	Max.	Mean	SD
log2_FRNT50	201	0	201	0,000	10,322	6055	2574
log2_SN	201	0	201	0,138	10,322	5585	1940

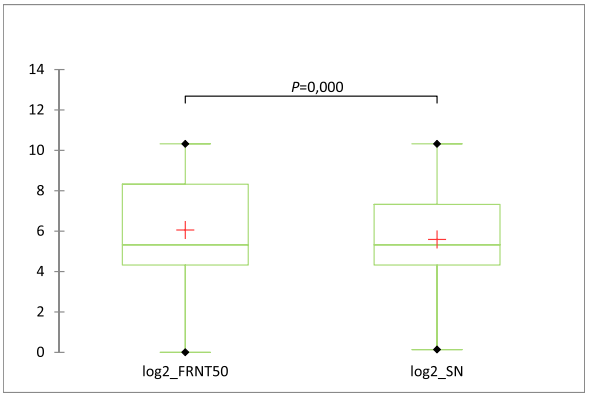

Fig. 1. Box plot presenting SN results with respect to the different FRNT50 titres. The non-parametric Wilcoxon test was used to compare sets of data. α = 0,05; an approximation has been used to compute the p-value (*P*). Results are expressed as $\log_2$.

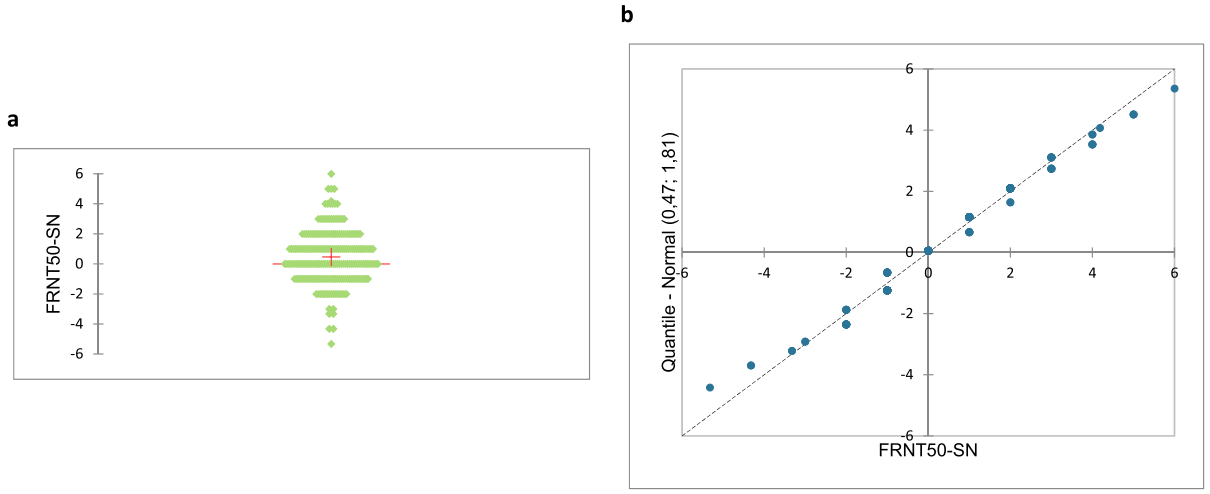

Fig. 2. Descriptive statistical analysis graphs from FRNT-SN comparison. a Scattergram; b Q-Q plot. Results are expressed as FRNT50 and SN $\log_2$ difference.

CRediT authorship contribution statement

Ilaria Puglia: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Marialuigia Caporale:** Writing – original draft, Methodology, Formal analysis, Data curation. **Claudia Casaccia:** Validation, Resources. **Fabrizia Valleriani:** Resources. **Romolo Salini:** Software. **Shadia Berjaoui:** Methodology, Formal analysis. **Cristina Marfoglia:** Validation. **Lucilla Ricci:** Validation. **Alessio Lorusso:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The authors declare no competing interests. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the IZSAM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34925.

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