Diagnostic accuracy for a plasma SARS-CoV-2 Nucleocapsid Protein method [version 1; peer review: awaiting peer review]

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Abstract

Background: The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) releases nucleocapsid proteins (NP) into the blood circulation in infected patients. We investigated whether plasma NP analysis could be used for diagnosing an infection and used for nosocomial screening.

Methods: We collected blood samples from patients admitted to the hospital during a period with reverse transcription polymerase chain reaction (RT-PCR) based-screening of patients for SARS-CoV-2. Retrospectively the SARS-CoV-2 NP plasma concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) method and used for an initial time course study to find the optimal time-point for sampling blood. Next, we estimated the diagnostic accuracy i.e. the clinical sensitivity and specificity at different plasma NP cut-off concentrations.

Results: The time course study revealed profiles with rapid or more slow declines in NP titers after the RT-PCR result. Nevertheless, in the time interval 0 – 7 days after the RT-PCR result, the NP concentration was always above the level of detection at 1.66 pg/ml suggesting that the diagnosis could be established in the time interval of 0 - 7 days. The median time gap between the plasma NP and RT-PCR results was 0.0 days (n = 1957, interval: -26 to + 21 days). Reducing the time gap to seven days, the clinical sensitivity was 90.0% (n= 60, 95% CI, 82.4% to 97.6%) at a specificity of 95.9% (n=1876, 95% CI, 95.0% to 96.8%). Curve analysis by receiver operation characteristics identified a cut-off concentration of 1.87 pg/mL NP as optimal resulting in a positive predictive value of 41.2%, a negative predictive value of 99.7% and a prevalence of 3.1%.

Conclusions: In conclusion, the NP method is acceptable for making the laboratory diagnosis of SARS-CoV-2, and an intended use of plasma NP as a prospective nosocomial screening method is
considered feasible.

**Keywords**
Plasma SARS-CoV-2 Nucleocapsid protein, SARS-CoV-2, diagnostic accuracy, clinical specificity, clinical sensitivity

This article is included in the Coronavirus collection.
Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel coronavirus which first appeared in Wuhan, China, in 2019. SARS-CoV-2 is an enveloped RNA virus that is distributed broadly among humans, other mammals and birds and that causes respiratory, enteric, hepatic and neurologic diseases and has presented an acute global challenge for both the public health, economy, and social life.5

Hopefully, the SARS-CoV-2 prevalence in the general population will decrease over the coming years. Nevertheless, it is envisioned that diagnosis of SARS-CoV-2 by systematic screening will still be required by the health authorities for several reasons. First of all, screening is the systematic application of a test or enquiry to identify individuals at sufficient risk of a specific disorder to warrant further investigation or direct preventive action amongst persons who have not sought medical attention on account of symptoms of that disorder.5 By analyzing over 350 studies, Sah et al.6 estimated that the percentage of SARS-CoV-2 infections that never developed clinical symptoms, and thus were truly asymptomatic, was 35.1% (95% confidence interval [CI]: 30.7 to 39.9%). At the time of testing, 42.8% (95% prediction interval: 5.2 to 91.1%) of cases exhibited no symptoms, a group comprising both asymptomatic and presymptomatic infections. Thus, a systematic screening may identify SARS-CoV-2 in asymptomatic subjects that otherwise would not have been tested. Thus, systematic screening opens for due diligence to introduce precautions that may reduce the spreading of SARS-CoV-2 in the general population.

Molecular and immunochemical-based methods can in principle analyze a plethora of bodily fluids e.g. the upper respiratory tract can be investigated with nasopharyngeal and oropharyngeal swab techniques, and the lower tract by collection of tracheal secretions. However, variations in sampling technique and the heterogeneity in the obtained material may compromise the quality of the analysis. In general, the clinical sensitivity and specificity of the rapid flow immunoassays are considered inferior to the more sensitive reverse transcription polymerase chain reaction (RT-PCR) based-methods.7–12

Interestingly, early studies of coronavirus replicating via infection of human cells revealed large amounts of NP antigen in the blood circulation.13–15 In contrast to swab material, plasma samples are a homogenous well-defined material and the most predominant type of sample in hospital laboratories, which may enable plasma SARS-CoV-2 diagnosis without extra cost or trauma to the patients.16–19 Furthermore, analysis of blood samples allows for a meaningful determination of a concentration by a quantitative method, and thereby also for definition of the appropriate specific diagnostic cut-off value of said method.

We hypothesized that screening of blood samples could be used to detect SARS-CoV-2 infections among patients being admitted to the hospital. In the present retrospective method comparison study, we estimated the diagnostic accuracy i.e. the clinical sensitivity and specificity of an ELISA-based plasma NP method as a potential prospective tool for nosocomial screening.

Methods

This retrospective study was checked for essential items as described in the guideline “standard for reporting of studies of diagnostic accuracy” (STARD criteria).20

Identification of eligible patients and blood samples

In the period from 1st - 30th November 2021 oro- and nasopharyngeal swab samples were obtained from patients admitted to the North Zealand Hospital, Hillerød, Denmark. To diagnose SARS-CoV-2 the samples were analyzed for the presence or absence of viral RNA with a RT-PCR method. As part of the standard procedure venous blood was also collected as requested by the clinicians for routine biochemical analysis. All plasma samples where P-Amylase were requested by the clinicians were collected and analyzed by an ELISA-based plasma NP method. P-Amylase is requested for all admitted patients at the emergency department and is a rare request from other departments. No other criteria were used to select or exclude samples for NP analysis. Several serial samples were obtained from the same patients and time course studies of changes in NP concentrations were then carried out. Paired NP and RT-PCR results were obtained, either with an either positive or negative time gap between the time of collection of blood samples for NP ELISA and pharyngeal swab for RT-PCR, respectively. The time course study was used to identify valid paired data within an optimal time gap. These paired data were used for calculating the final diagnostic accuracy i.e. the clinical sensitivity and specificity of the NP assay using the RT-PCR as the reference method. The paired data analysis was carried out by a person without prior influence or knowledge of the results from the RT-PCR and NP analysis.

RT-PCR reference method

Within 2-3 hours oro- and nasopharyngeal swab samples were subjected to RNA isolation and RT-PCR amplification. In brief, viral RNA and human RNA/DNA were isolated from 0.2 mL inactivating NEST buffer (Wuxi NEST
Biotechnology Co, Ltd., Jiangsu, China) using a CE-marked method (MagMAX™ viral/pathogen nucleic acid kit). The isolation was performed on an automated flow Robot system (Flow Robotics A/S, Copenhagen, Denmark) and the KingFisher™ Flex purification system (Thermo Fisher Scientific, Darmstadt, Germany).

Five µL RNA elution buffer was transferred to 15 µl TaqMan Fast Virus 1-step master mix (Thermo Fisher Scientific, Darmstadt, Germany) containing 200 mmol/L and 400 mmol/L Envelope (E) gene and Ribonuclease protein (RNase P) hydrolysis probes and primers, final concentrations respectively. The probes were dual labelled with 6-carboxyfluorescein and black hole quencher (E gene) and 6-carboxy-rhodamine and black hole quencher (RNase P) (Merck KGaA, Darmstadt, Germany). RT-PCR was performed at 55°C for 10 min, 95°C for 1 min, and 38 cycles at 95°C for 10 sec and 60°C for 30 sec (AriaDX instruments, Agilent Technologies, USA).

An arbitrary threshold in signals was set to remove background amplification noise. Exponential curve signals above the background noise with Ct values < 32 were considered positive. Samples with Ct values of 32 – 38 were re-analyzed. The PCR amplification efficiency was > 96% with a level of detection of 13 ± 25 (mean ± standard deviation [SD]) DNA copies per reaction. Internal negative and positive E gene synthetic RNA controls (Twist Bioscience, South San Francisco, CA, USA) were included in every run. The intra-analytical precision for the E gene and RNAse P methods were 6.3% at Ct values of 13.8 ± 0.86 and 21.3 ± 1.35 (mean ± SD, n =14), respectively. Analytical precision of the entire laboratory set up was estimated by adding MS-2 phages (American Type Culture Collection) to NEST buffer followed by RNA isolation and RT-qPCR. The intra-analytical precision for the Ct value was 2.2% (Ct mean 22.37 ± 0.50, n = 16).

The diagnostic performance of the RT-PCR reference method was validated by analyzing a blinded batch of swab samples collected by the Department of Clinical Microbiology (Herlev Hospital, Denmark). In brief, the blinded result of 21 positive and 73 negative SARS-CoV-2 were 100% in concordance with the result initially found by the external laboratory. SARS-CoV-2 positive samples were sent daily to an independent laboratory for mutation analysis (Department of Clinical Microbiology, Herlev Hospital, Denmark).

NP test method
Routine blood samples were sent to the local Department of Clinical Biochemistry by a Tempus 600 pneumatic tube system (Timedico A/S, Bording, Denmark). After all the requested biochemical analyses were carried out, the centrifuged plasma samples were kept for one day at 4°C. The plasma samples were frozen at -20°C and later thawed batch-wise for NP analysis. The NP analysis was carried out by a person without knowledge of the RT-PCR results.

For the plasma NP analysis, a BEP 2000 Advance System (Siemens Healthineers Inc) was programmed according to the instructions given by the local distributor (Solsten Diagnostics International, Aarhus, Denmark). Up to 12 strips of each 8 wells precoated with antibody to SARS-CoV-2 NP were mounted in each 96-well frame. First, 50 µL of biotin-conjugated antibody was added to each well and then directly supplemented with 50 µL of either internal NP calibrator or plasma. The wells were incubated for 1 h at 37°C, washed five times, incubated with 100 mL/well peroxidase-conjugated streptavidin for 30 min at 37°C, washed five times and then incubated with the provided substrate for 15 min at 37°C. The chromogenic enzyme reaction was stopped by photometrical measurements of the absorbance. Standard curves of five internal calibrators (0 and 160 pg/mL) were used as defined by the manufacturer. The cut off value is prespecified by the manufacturer. The present study optimal cut-off values for the NP ELISA results were determined in an exploratory fashion by receiver operating characteristic (ROC) curve analysis.

Ethics
The present quality control study of the NP test method did not imply extra blood sampling from the patients. The obtained results had no impact on clinical care decisions, and no clinical information (except age and sex) was collected. Accordingly, the present quality control study of two methods required no written or oral permission from the patients.

Analysis of data
Csv-files were downloaded from the Oracle database PKLABKA (LABKA, HealthCare Denmark – CSC A/S, Odense, Denmark) using the SQL Developer software version 11.0.4.1774 (Allround Automations V.O.F, Enschede, Netherlands). Only plasma samples where P-amylose was requested by the clinicians was selected for later NP analysis (please see prior section). The SQL algorithm used the plasma sample number from the P-amylose and NP analysis to find the date for the collection of the blood sample, the age of the patient and sex. The RT-qPCR results was extracted from the Clinical Microbiology Laboratory database system. To ensure full data security each patient was equipped with one unique random number. Pairing of NP and RT-qPCR results originating from the two databases was carried out with standard EXCEL 2016 software by the use of the unique random number. Patients without a paired NP and RT-qPCR
result was discarded. Please see Table 1 for description and number of individuals. The author S. K was responsible for accessing the PKLABKA database, and Tina Proft Larsen (acknowledgement) for providing the RT-qPCR results.

### Statistics

The statistical uncertainty of the estimates is reported as mean ± SD, number of measurement (n). A p < 0.05 was considered statistically significant. The 95% confidence intervals of percentages (\(p\)) were calculated as 
\[
\pm 1.96 \sqrt{\frac{p(1-p)}{n}}.
\]

### Power analysis

The power and intended sample size were calculated a priori as requested by the STARD criteria by following the Clinical and Laboratory Standards Institute (CLSI) guideline EP-24. The manufacturer claimed a clinical sensitivity of 30/32 subjects, or 93.75% (95CI: 79.19% - 99.23%) for results analyzed \(\leq\) 3 days from start of symptoms, and a specificity of 649/649 subjects giving 100% (95CI: 99.43-100.00%) when using a cut-off of 2.97 pg/ml. The intended minimum subjects with an error < 5% was estimated to 90 subjects. The screen positive rate of approximately 4.8% in November 2021 consequently required at least 1875 (90 x 100/4.8) samples. The study was carried out with collection of the required number of samples before the blinded analysis was carried out.

### Results

As shown in Figure 1, the time course study of plasma NP concentrations showed considerable individual variations among the SARS-CoV-2 positive patients. Overall, there was a decline in NP concentrations and a time gap of 0-7 days was identified as a window for collecting samples for NP analysis.

A total of 3334 plasma samples were analyzed with the NP test method. Among the 3334 plasma samples 2653 unique patients were identified; this cohort consisted of 1415 women and 1238 male subjects with 13 subjects excluded due to lack of identification, as shown in Table 1.

A total of 1957 unique patients with a paired NP and PCR results were identified. This cohort included 63 and 1894 patients with a positive or negative RT-PCR result, respectively. Hence, an initial screen positive rate of 3.2% (63/1957) could be calculated.

The median time lag between the RT-PCR and NP analysis date were 0.0 days (\(n = 1957\), lag interval: -26 to +21 days). Since 83% and 98% of the present paired data were collected within 0-3 and 0-7 days apart, respectively, we performed an initial ROC curve analysis without exclusion of any paired data set.

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**Table 1. Description of number of collected plasma samples and patients.** In the period from 1st - 30th November 2021 oro- and nasopharyngeal swab and blood samples were obtained from patients admitted to the North Zealand Hospital, Hillerød, Denmark. To diagnose severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) the swab samples were analyzed for the presence or absence of viral RNA with a reverse transcriptase-polymerase chain reaction (RT-PCR) method and the plasma was analyzed for nucleocapsid protein (NP) content with an enzyme-linked immunoassay (ELISA) method.

<table>
<thead>
<tr>
<th>Total</th>
<th>Women</th>
<th>Men</th>
<th>Excluded*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of plasma samples</td>
<td>3334</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Unique patients (n) for NP</td>
<td>2653</td>
<td>1415</td>
<td>1238</td>
</tr>
<tr>
<td>Median age, years</td>
<td>67.9</td>
<td>66.6</td>
<td></td>
</tr>
<tr>
<td>Age interval (17.2-105.5)</td>
<td>(0.6-96.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paired NP and PCR results (n)</td>
<td>1957</td>
<td>1028</td>
<td>928</td>
</tr>
<tr>
<td>Median age, years</td>
<td>64.0</td>
<td>63.9</td>
<td></td>
</tr>
<tr>
<td>Age interval (17.2-105.5)</td>
<td>(0.6-96.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unique patients with a:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Positive RT-PCR result</td>
<td>63</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>- Negative RT-PCR result</td>
<td>1891</td>
<td>997</td>
<td>894</td>
</tr>
</tbody>
</table>

*Data excluded due to time-lag between test-results.
As shown in Figure 2, nine different NP cut-off values ranging from 0.83 pg/mL to 116.6 pg/mL were plotted. The sensitivity and specificity changed from 59–95% and 21–100%, respectively, with the nine different cut-off values. After comparing the distance from the nine data points to the X-Y line, the cut-off value of 1.87 pg/mL was selected. This cut-off gave an initial maximal sensitivity and specificity of 86% (54/63) and 95% (1808/1894), respectively. Selection of paired data with a maximal time gap between PCR and NP measurement of seven days finally adjusted the sensitivity and specificity to 90.0% (n = 60, 95% CI, 82.4% to 97.6%) and 95.9% (n = 1876, 95% CI, 95.0% to 96.8%), respectively.

Using the RT-PCR results as a reference this resulted in a positive predictive value of 41.2%, a negative predictive value of 99.7% and a prevalence of 3.1%.

**Discussion**

In the present study we first estimated an acceptable time gap for including plasma samples for NP analysis. The typical course of the NP concentrations was an initial high titer followed by a gradual decrease with only a few patients showing deviations from this pattern. We identified 0-7 days as an optimal time gap. This finding is in accordance with the study by Thudium et al., where the diagnostic accuracy of the NP method was shown to be strongly dependent on the time gap.
from the timepoint for the first PCR positive swap. The higher sensitivity after 4-7 days may reflect the prior time needed for invasion, synthesis, and release of N-antigens into the bloodstream upon lysis of the human epithelial cells.16 Furthermore, it could be speculated that the decrease or delta-value of NP could predict patient outcome. Moreover, the plasma NP may indicate that epithelial cells infected with SARS-CoV-2 are actively making virus proteins. Thus, a patient without NP - or with rapidly decreasing NP - could be viewed as non- or minimally contagious and could be withdrawn from an isolation protocol.

Using ROC curve analysis for setting the cut-off value with a time gap of seven days, the clinical sensitivity was 90.0% (n= 60, 95% CI, 82.4 % to 97.6%) with a clinical specificity of 95.9% (n=1876, 95% CI, 95.0% to 96.8%). The study found a positive and negative predictive value of 41.2% and 99.7%, respectively, and a prevalence of 3.1%. The present screen-positive rate was obtained in November 2021 at the Hospital of North Zealand. In the same period the PCR screen-positive rate in the capital area of North Zealand was initially 3.4 % in week 44 and increased to 4.8% by the end of week 48. This period was covered 98.9-100.0% (binomial 95% confidence interval) by the Delta variant B.1.617.2 containing the L452R mutation in the Spike protein.25,26 The high negative predictive value (99.7%) for the NP method could be used to rule out SARS-CoV-2 and thereby lead to reduction in the need for swab sampling including PCR analyses and/or suspension of quarantine of suspected SARS-CoV-2 patients.

The a priori specified intended sample size for the claimed clinical sensitivity was 90 positive SARS-CoV-2 subjects. However retrospectively 60 positive subjects were included after the study period. Recalculating with the present sample size of 60 subjects increases the p value to avoid a type II error from 0.05 to 0.07. Thus, in 7% of the cases the hypothesis will be untrue in the full study population. The 7% chance of a type II error is considered acceptable for an initial pilot study. In comparison, the CE-labelled NP assay claimed a clinical sensitivity of 93.75% (30 subjects out of 32) and 100.00% (38/38) when the NP test was carried out ≤ 3 days and 4-7 days from start of symptoms, respectively, with a cut-off value of 2.97 pg/mL.22

In the study by Thudium et al.16 a group of 99 inpatients and 505 outpatients was evaluated using a cut-off value of 10 pg/mL. When blood was collected between 0-6 days a specificity of 99.8% was found in the group of out-patients, and the sensitivity was 81.4% and 92.9% for the group of out- and inpatients, respectively. Taken together, the present study and the study by Thudium et al.16 shows that the clinical accuracy depends on the time gap and can be adjusted by an optional cut-off value.

The present study used the E gene as the target for detecting SARS-CoV-2 RNA.21 Studies of the analytical and clinical performance of RT-PCR methods based on E gene supports the notion that RT-PCR of the E gene is an acceptable reference method. However, a systematic review of 34 studies enrolling 12.057 SARS-CoV-2-19 confirmed cases reinforces the need for repeated testing in patients with suspicion of SARS-CoV-2 infection given that up to 54% of SARS-CoV-2 patients may have an initial false-negative RT-PCR.35 Taken together,35 the relatively high false negative rates of SARS-CoV-2 RT-PCR testing need to be accounted for in clinical decision making, epidemiological interpretations, and when using RT-PCR as a reference for other tests.

The NP ELISA method could contribute to reduce the risk of nosocomial SARS-CoV-2 infection. A suitable laboratory diagnostic method for NP in blood samples must provide an analytic limit of detection close to 1 pg/mL corresponding to 20 fM. Also, it must be sufficiently robust and specific to avoid false positives even when used for analysis of undiluted blood samples. However, many hospital laboratories must handle many samples with a minimum of manual resources and short turn-around times. These requirements are often solved by an automated continuous single-cell reaction of beads coated with specific antibodies. The present NP ELISA format requires manual handling and collection of a batch of plasma samples before a run can be initiated. Thus, the overall economy and turn-around times of implementing nosocomial screening with the NP method should be considered.

In conclusion, the retrospective comparison of the plasma NP test against the RT-PCR reference method shows that the NP method has an acceptable diagnostic accuracy. Thus, the NP method is suitable for diagnosis of SARS-CoV-2. Finally, the NP method could be used for prospective nosocomial screening for SARS-CoV-2.

Data availability

Underlying data
This project contains the following underlying data:

- Data file 1. SARS-CoV-2 Nuclecapsid and RT-PCR results.csv
- Data file 1. README.file.txt

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication)

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References


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