The Importance of SARS-CoV-2 N-Ag Serodiagnostics for the Management of COVID-19 Pneumonia in Hospital Settings

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Research

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Abstract

We report evaluation of the SARS-CoV-2 nucleocapsid antigen (N-Ag) and respective antibodies as diagnostic markers in pneumonia patients. The study was conducted at the height of COVID-19 pandemic in Moscow, Russia. It included 425 emergency patients with clinical signs of COVID-19 pneumonia, of which 280 (66%) were positive for either serum N-Ag and/or its respective antibodies. We demonstrate the total prevalence of N-Ag seroconversion in SARS-CoV-2-associated pneumonia patients within 3-5 days after hospital admission. The results indicate high feasibility of SARS-CoV-2 serodiagnostics in emergency patients.

Introduction

Immunochemical detection of antigens in blood is widely used in the diagnostics of infectious diseases. The antigenemia tests are usually targeted at blood-borne pathogens (notably chronic viral infections including CMV, HBV, HCV and HIV [1–3]). In respiratory diseases, the penetration of the pathogen components (nucleic acids or proteins) into non-respiratory body fluids is likely, but their detection is usually considered of minor value because of the focal nature of the pathogenesis and its strong association with mucous immunity. Despite that, certain respiratory infections can be efficiently diagnosed and monitored by detection of corresponding antigens in the blood; the examples include galactomannan in pulmonary aspergillosis [4] and *L. pneumophila* antigen in urine of Legionnaires' disease patients [5]. In addition, the use of serological immunoassays for the management of virus-associated nosocomial pneumonias in intensive care unit patients has been reported [4, 6]. Serum nucleocapsid antigen (N-Ag) of SARS virus was described in SARS-associated pneumonia by Che et al [7]

In this article, we report evaluation of the SARS-CoV-2 nucleocapsid antigen (N-Ag) and respective antibodies as diagnostic markers and demonstrate the total prevalence of N-Ag seroconversion in SARS-CoV-2-associated pneumonia patients.

Patients And Methods

The study was carried out at the Federal Center of Brain Research and Neurotechnologies during the spring of 2020. At the height of the COVID-19 pandemic, the in-patient care facilities of the Center were redesigned for the emergency hospitalization and management of the SARS-CoV-2-associated pneumonia patients. The study involved patients with clinical signs of COVID-19 pneumonia (n = 425) flown by ambulance services from different districts of Moscow. The key criterion for the emergency admission was fever with a combination of two or more clinical signs including body temperature of $\geq$ 38.5 °C, respiratory rate of $\geq$ 30 breaths per minute and/or SpO_2 of $\leq$ 93% [8]. Serum samples for the study were collected from each patient at least twice; sampling at the admission and at the discharge was carried out concomitantly with the mandatory procedure for routine blood tests. The in-patient care lasted 20 ± 2 days. Criteria for the discharge included a reduction in C-reactive protein levels at WBC counts within the normal range (above 4.0×10^9 L) and, notably, a clear tendency towards regression of

characteristic signs revealed by computer tomography: the absence of new ground glass opacities, a decrease in the severity of the corresponding changes in the lung tissue and/or a decrease in the volume or degree of consolidation of the ground glass opacities (no more than three, each within 3 cm along the maximal dimension) [9]. The qualitative RT-PCR detection of SARS-CoV-2 RNA in nasopharyngeal swabs was carried out at the admission with the use of SARS-CoV-2/SARS-CoV Multiplex Real-Time PCR Detection Kit Reg.No. 2020/9948 (DNA-Technology LLC, Moscow, Russia; analytical sensitivity 10 copies per amplification tube, diagnostic sensitivity (95% CI) – 100% (95.6–100%), diagnostic specificity (95% CI) – 100% (96.7–100%)) according to the manufacturer's protocol.

SARS-Cov-2 nucleocapsid antigen (N-Ag) coding sequence (NCBI accession number 045512.2) was cloned by NdeI/XhoI into pET-30b(+) vector, Novagen (EMD Millipore); the plasmid was expanded in *E. coli* Top10 and transformed into *E. coli* Rosetta (DE3) for N-Ag expression.

The conditions were optimized for 37 °C bioreactor fermentation in 3000 mL of rich media (yeast extract, bacto peptone, glucose and trace salts). After 12 h incubation, the culture was induced with 2.5 mM imidazole for 4 h. The obtained biomass was resuspended in phosphate buffered saline (PBS) in a 1:3 (w/v) proportion and disrupted using APV-2000 homogenizer (Spx Flow, USA) at 1200 bar. The solution was clarified by centrifugation at 12,000 g. The insoluble fraction (inclusion bodies) containing the target protein was washed sequentially with PBS, 2% Triton in PBS and a fresh portion of PBS to remove the residual detergent. The protein was dissolved in 8 M urea with 250 mM NaCl and 50 mM phosphate, pH 10.0. The solution was incubated at + 4 °C overnight and centrifuged at 15,000 g; the collected supernatant was filtered and supplemented with 20 mM imidazole to prevent the non-specific binding of impurities. The polyhistidine-tagged N-Ag protein was purified by immobilized metal affinity chromatography using a nickel column (High Density Nickel #6BCL-QHNi, ABT, Spain) equilibrated with the urea buffer. The protein was eluted with 250 mM imidazole buffer and filter-sterilized.

Serum IgG antibodies against nucleocapsid antigen (N-IgG) were detected by solid-phase enzyme immunoassay. Briefly, the recombinant full-length SARS-CoV-2 nucleocapsid antigen produced in *E. coli* (XEMA, Moscow, Russia) was coated onto the surface of polystyrene microwells. The sera pre-diluted 100-fold in the ELISA buffer (0.1% Tween-20 and 1% hydrolyzed casein in 0.1 M PBS) were placed in the microwells for 30 min at 37 °C. After 3 washes with 0.1% Tween-20 in 0.9% sodium chloride, the wells were exposed to the conjugate of murine monoclonal antibodies XG78 against human IgG (gamma chain) with horseradish peroxidase (XEMA) for 30 min. After 5 washes with 0.1% Tween-20 in 0.9% sodium chloride, the bound enzyme was revealed by addition of the substrate-chromogenic mix (TMB substrate, XEMA). The color development was stopped by 5% sulfuric acid and the optical density (OD) at 450 nm was measured in a plate reader (Multiskan MC, Thermo Labsystems). The internal controls (stabilized human serum containing specific IgG antibodies) were included in all microplates to calculate the positivity threshold OD for each run individually. The results are expressed as positivity indexes (calculated as OD for a sample of interest related to OD for the internal control).

Serum IgM antibodies against nucleocapsid antigen (N-IgM) were detected by reverse solid phase enzyme immunoassay. Briefly, murine monoclonal antibody against the mu chain of human IgM (clone X616, XEMA) was adsorbed on the surface of polystyrene microwells. The dilutions of sera (prepared in the same way as for the IgG assay) were placed in the microwells for 30 min at 37 °C. After 3 washes with 0.1% Tween-20 in 0.9% sodium chloride, a working dilution of the recombinant full-length SARS-CoV-2 nucleocapsid antigen conjugated with horseradish peroxidase in the ELISA buffer was placed in the microwells for 30 min at 37 °C. After 5 washes with 0.1% Tween-20 in 0.9% sodium chloride, the bound labeled antigen was detected by TMB substrate and OD reading. The calculation of positivity index (similarly with the IgG assay) was performed by using the internal IgM + control.

Serum N-antigen (N-Ag) determination was performed by the two-site solid-phase sandwich method using monoclonal antibodies (mAbs) generously gifted by Hytest Ltd, Turku, Finland. The microwells were coated with the capture mAb (clone NP1510, HyTest). Serum samples or assay calibrators (solutions of nucleocapsid antigen in donor serum in the range 20-2000 pg/ml) were incubated with a working dilution of HRP-labeled tracer monoclonal antibody (clone NP1517, HyTest) in ELISA buffer with the addition of heterophilic immunoglobulin elimination reagent (10 ug/ml, HIER-E-010, Fapon Biotech, China) for 2 h at 37 °C under continuous 600 rpm shaking in a PST-60HL-4 shaking incubator (Biosan, Latvia). The detection was performed with the use of TMB substrate and OD reading. The N-Ag concentrations were determined by calibration curve method using the DataGraph data reduction software (Visual Data Tools, Inc.). N-Ag concentrations exceeding the upper limit of calibration curve (2000 pg/ml) were shown as 2222 pg/ml in calculations and graphic presentation. The samples with OD readings lower than 20 pg/ml, lacking resolution from the zero calibrator, were conventionally assigned 15 pg/ml values in the presented data.

The data were processed in GraphPad Prism 8.0 (GraphPad Software, Inc.) and Microsoft Excel 2016 software.

The reference ranges were determined by analysis of serum samples collected from healthy donors (n = 250) before Dec 2019. In both assays, the threshold (cutoff) values were set to attain full specificity (none of the donors being considered positive).

The protocol for the study was reviewed and approved by the Local Ethics Committee at the Pirogov Russian State Medical University (Meeting No. 194 of March 16 2020, Protocol No. 2020/07); the study was conducted in accordance with the Declaration of Helsinki.

Results And Discussion

The immunoassay-based detection of serum N-Ag in combination with its respective antibodies confirmed COVID-19 in 280 patients (66%) of the studied cohort. RT-PCR analysis of nasopharyngeal swabs confirmed COVID-19 in 76% of the patients; the interception constitutes 63% and corresponds to the concordance of 79%.

Several patients (n = 21, 5%) were identified as SARS-CoV-2 negative by both RT-PCR tests and serodiagnostics, and most likely represented cases on non-SARS-CoV-2-associated pneumonias. The small number of such patients is explained by the fact that the study was carried out at the peak of the pandemic.

According to the data, N-Ag antigenemia is characteristic of the majority of patients with severe COVID-19 (63%).

The disease phase-related dynamics of serum N-Ag levels are shown in Fig. 1. At the discharge, most of the patients N-Ag positive at the admission (104 of 116, 90%) showed serum N-Ag levels below the lower limit of the calibration curve (< 20 pg/ml; 104 of 116, 90%). However, some patients were clearly positive for serum N-Ag even at the discharge.

By the time of discharge, seroconversion was observed in most of the patients N-Ag positive at the admission (108 of 116, 93%), although the degree of seroconversion varied considerably. To determine seroconversion patterns, we plotted serum N-Ag levels with two isotypes of antibody response for individual cases, dividing them in two subgroups by Ab-seropositivity at the admission (Figs. 2a and 3a *vs.* 2b and 3b). The results indicate reciprocal patterns for N-Ag and respective antibodies, which is characteristic of classical seroconversion. The patients Ab-seronegative at the admission (presumably being at the earlier stage of the disease) showed more distinct patterns of classical seroconversion. The IgG-antibody patterns were more pronounced than the IgM-antibody patterns in both groups.

Co-detection of N-Ag and anti-N-Ag antibodies in 92 (18%) of 503 serum samples demonstrates that the epitope recognized by immunoassay pair used for the antigen determination is at least not fully overlapped (masked) by the human antibody response.

For a subcohort of 20 patients, we had the opportunity to evaluate seroconversion within a shorter period of several days. According to the results shown in Fig. 4, in the majority of cases the switch from Ag + Ab- to Ag-Ab + status proceeded within 3–5 days after the emergency admission.

Overall, the results indicate high relevance of combined serological tests for SARS-CoV-2 N-Ag and the antibodies to SARS-CoV-2 antigens as applied to the infectious pneumonia emergency patients at the admission, since:

- sensitivity and specificity of the serological test for N-Ag is comparable to that of the RT-PCR analysis of nasopharyngeal swabs, and the use of serology tests in combination with PCR tests for SARS-CoV-2 provides a significant increase in the overall sensitivity;
- processing of blood samples is safer for the personnel due to the negligible presence of SARS-CoV-2 infectious particles in the blood, by contrast with nasopharyngeal swabs;
- serological tests are generally faster than RT-PCR tests (when accounting for the full cycle of biomaterial processing and sample preparation), which accelerates the determination of SARS-CoV-2

status for a prompt decision on the management mode and isolation measures; in addition, blood serum analyses are easier to automate;

- collection of blood samples for serodiagnostics of SARS-CoV-2 in hospital settings is not an additional invasive procedure, but is performed as a part of the mandatory primary examination immediately upon the admission (as well as of the final examination upon the discharge from the hospital).

According to the results of the study, hospitalization of the patients with SARS-CoV-2-associated pneumonia at the height of pandemic most frequently occurred before the onset of seroconversion (i.e. against the background of detectable serum N-Ag concentrations). In the majority of patients, the antigen prevailed at the time of admission and was "replaced" with respective antibodies by the time of discharge. Nevertheless, in some cases, by the time of emergency hospitalization, the level of antibodies to N-Ag in the patient's blood was already high, probably reflecting either delayed hospitalization or individual variation in the COVID-19 pathogenesis.

Overall, the obtained results indicate high feasibility of SARS-CoV-2 serodiagnostics in the emergency patients (along the lines of ‘rapid tests’ Capillus HIV-1/HIV-2®, Determine HIV-1/2®, etc. currently proposed as a gold standard of HIV diagnostics instead of the more expensive and time-consuming western blot analysis [10]). It should be noted that false-positive results of SARS-CoV-2 serodiagnostics may appear in patients with high levels of rheumatoid factor or HAMA antibodies, which underscores the significance of personalized comprehensive diagnostics for the patients with severe COVID-19.

Declarations

Ethics approval and consent to participate: The study protocol was reviewed and approved by the Local Ethics Committee at the Pirogov Russian State Medical University (Meeting No. 194 of March 16 2020, Protocol No. 2020/07); the study was conducted in accordance with the Declaration of Helsinki.

Consent for publication: Not applicable.

Availability of data and materials: The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: YSL designed the study, performed the sampling, conducted molecular studies and drafted the manuscript, OVL and AGG performed the clinical examinations and sampling, conducted biochemical and molecular studies, AVP conducted molecular studies and drafted the manuscript, VVB

and DVR designed the study and drafted the manuscript. All authors read and approved the final version of the manuscript.

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Figures

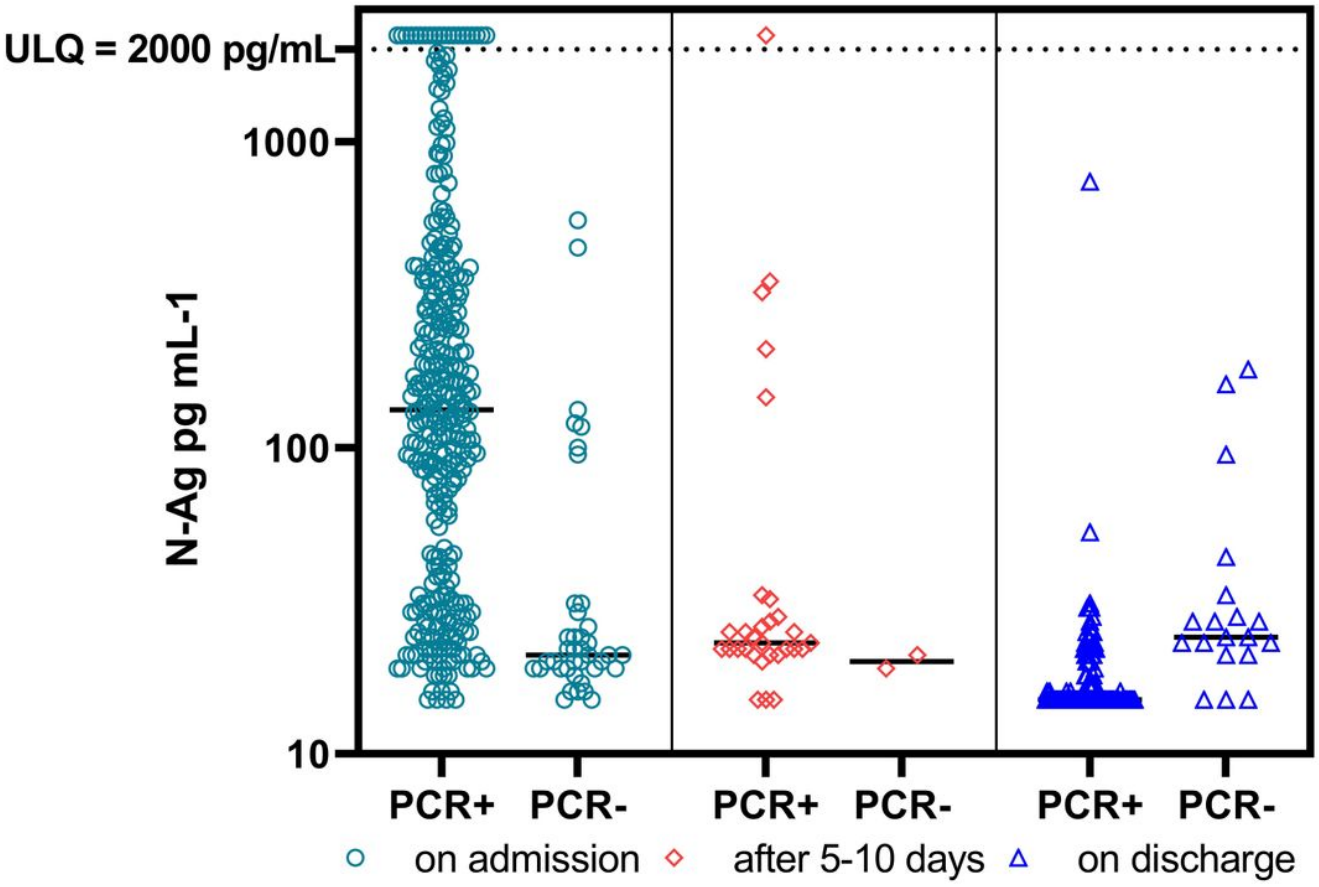

Figure 1

Dynamics of serum N-Ag levels in the studied cohort of hospital patients. ULQ, upper limit of quantification. The 'PCR+' and 'PCR-' indications refer to the patient's status at the admission.

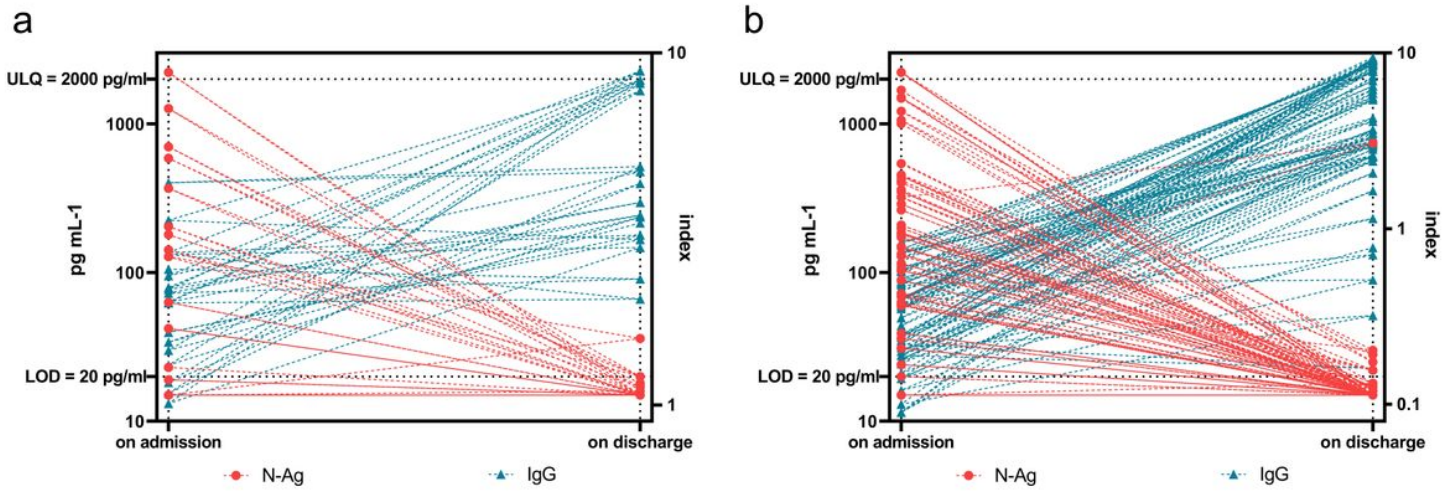

Figure 2

Dynamics of serum N-Ag levels vs. respective IgG antibodies in patients IgG-seropositive at the admission (a) and patients IgG-seronegative at the admission (b). Vertical axes correspond to Ag concentration (on the left) and Ab index (on the right). ULQ, upper limit of quantification; LOD, (lower) limit of detection.

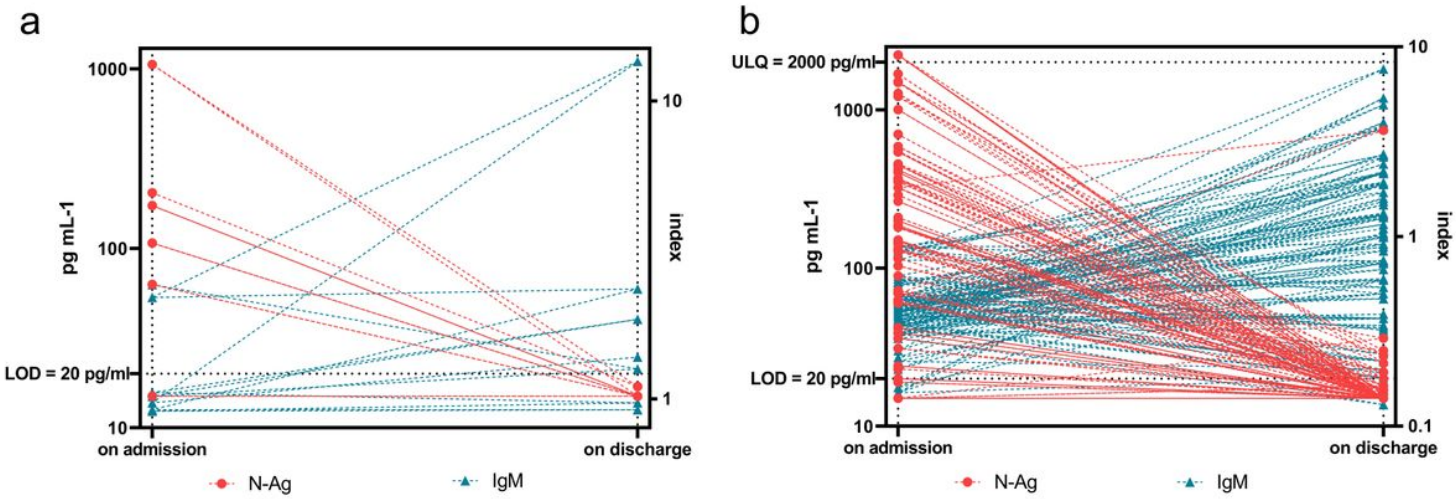

Figure 3

Dynamics of serum N-Ag levels vs. respective IgM antibodies in patients IgM-seropositive at the admission (a) and patients IgM-seronegative at the admission (b). Vertical axes correspond to Ag concentration (on the left) and Ab index (on the right). ULQ, upper limit of quantification; LOD, (lower) limit of detection.

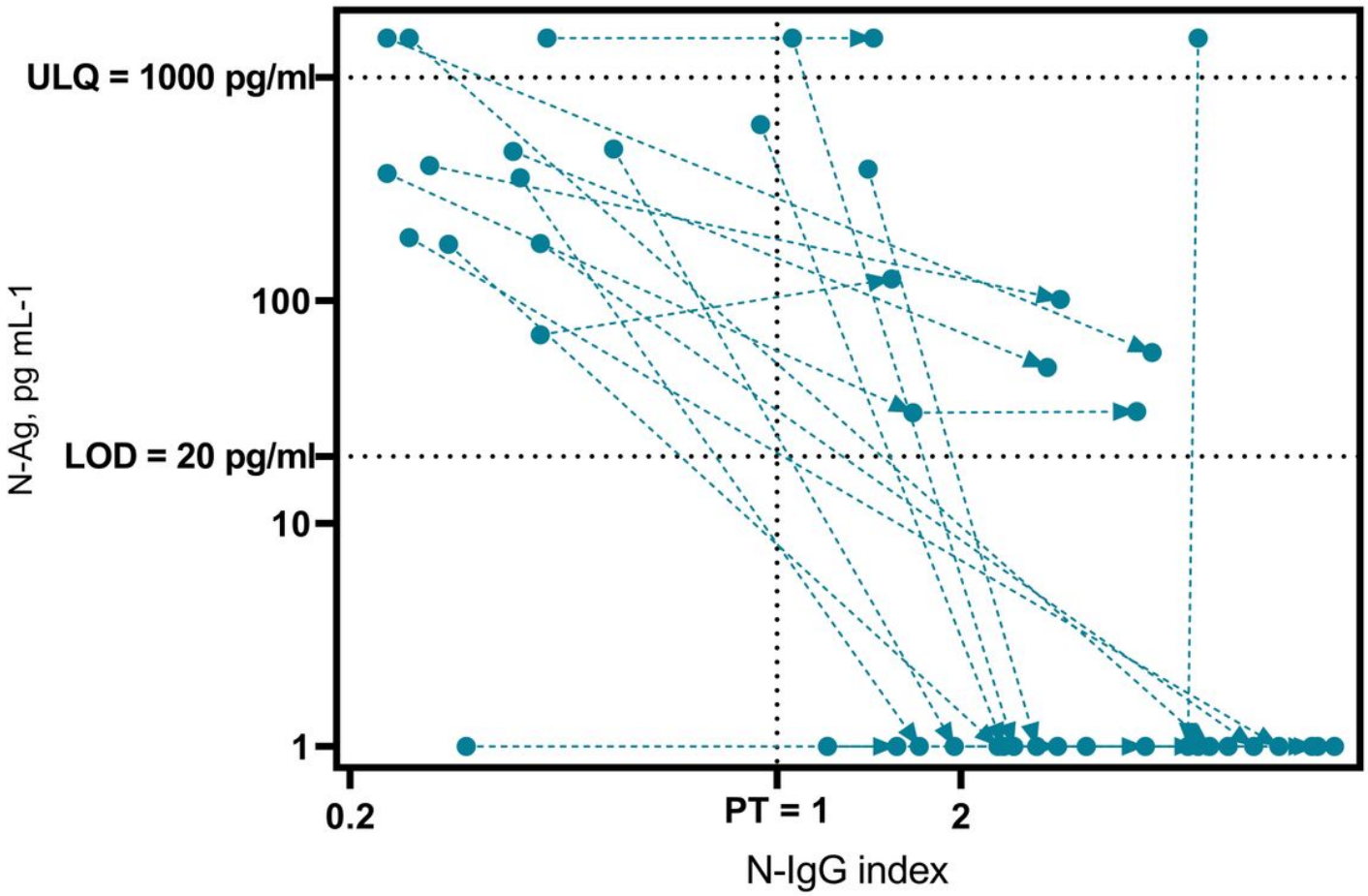

Figure 4

Individual patterns of seroconversion in the course of 3-5 days after the admission. ULQ, upper limit of quantification; LOD, (lower) limit of detection; PT, positivity threshold.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Lebedinetalrawdata20.xlsx