



Association of Anti-N and -S Seroprevalence in Asymptomatic, Mildly Symptomatic, and Symptomatic SARS-COV-2 Natural Infection

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ABSTRACT

Backgrounds: SARS-COV-2 infection is not always correlated with protection. Antibody seroprevalence in unvaccinated individuals, which is usually measured by N-specific antibodies, is not necessarily correlated with protection, while antibodies against S protein show a better correlation with protection due to its neutralizing epitopes. In this study, we tried to improve our conception of the hidden perspective of SARS-COV-2 in epidemiological reports and investigate anti-S antibody prevalence among anti-N antibody-positive asymptomatic and mildly symptomatic patients.

Materials & Methods: Blood samples were collected from asymptomatic or mildly symptomatic volunteer participants and symptomatic hospitalized patients with negative PCR results from May 30 to June 17, 2020. Detection of SARS-COV-2 antibodies was done using an ELISA kit targeting N or S protein.

Findings: Totally, 716 samples from volunteer participants and 81 samples from symptomatic hospitalized patients with negative PCR results were evaluated. The test performance-adjusted seroprevalence (%95 CI) of SARS-COV-2 antibody was 17.3% (8.8-25.8%) for anti-N IgG in volunteers and 25.5% (12.8-39.7%) for anti-N and anti-S IgM in hospitalized patients. Among anti-N IgG positive infected individuals, 49.2% (21.4 and 78.8%) were anti-S antibody positive.

Conclusion: The results showed that SARS-COV-2 infection sometimes occurs in individuals without symptoms or with mild symptoms, but in more than half of them, the produced antibody is not protective. The findings of hospitalized patients showed that the combination of IgM assay with real-time PCR improved the disease diagnosis by more than 25% in cases with negative molecular test results.

Keywords: Antibody, COVID-19, Epidemiology, SARS-COV-2, Seroprevalence.

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Introduction

The latest member of the Coronaviridae family, SARS-COV-2, is the causative agent of a respiratory disease worldwide [1]. The coronavirus disease 2019 (COVID-19) has caused a spectrum of illnesses which are divided into three levels: asymptomatic or mildly symptomatic disease, symptomatic disease which requires medical care, and severely symptomatic disease with serious conditions and organ failure, which requires hospitalization in ICU (intensive care unit) [2, 3]. Due to the similarity of COVID-19 common symptoms such as fever, fatigue, and cough [2, 4] with other respiratory diseases like Influenza [5], the detection of confirmed COVID-19 cases in official reports is based on molecular tests [6], these cases do not include asymptomatic or mildly symptomatic patients who don't refer to medical centers and also patients with false-negative RT-PCR results, while the infection is contagious in all of these conditions [7, 8].

Antibody assay against SARS-COV-2 N protein, as a highly antigenic and widely expressed protein during the infection, could retrospectively show the infection status in the community, even in asymptomatic cases. It may also help diagnose the disease in cases with false-negative molecular test results. The presence of antibodies against N protein does not confirm protection as this protein does not induce the production of neutralizing antibodies; thus, asymptomatic or mildly symptomatic patients and also patients with false-negative RT-PCR results may produce anti-N antibodies, as noted in epidemiological reports, but may not be immune against re-infection. Due to the presence of neutralizing epitopes in S glycoprotein, anti-S antibodies to some extent could induce protection against re-infection in individuals. Therefore, the status of anti-S antibodies among anti-N antibody positive individuals reported in epidemiological studies could

help clarify the status of herd immunity to SARS-COV-2 to make decisions about vaccination programs.

Objectives: In the present study, to improve our conception of herd immunity to SARS-COV-2 without vaccination or following discontinuation of the general vaccination program, anti-N and anti-S antibody status was evaluated in asymptomatic or mildly symptomatic volunteer participants and severely symptomatic hospitalized patients with negative PCR results before vaccination. The role of job status and social behaviors in antibody prevalence was also evaluated.

Materials and Methods

Study design and participants: Peripheral venous blood samples were collected from two groups of people in Tehran and Mashhad cities from May 30 to June 17, 2020: (1) asymptomatic, mildly symptomatic, or symptomatic volunteer participants who did not refer to medical centers due to COVID-19 symptoms and (2) severely symptomatic hospitalized patients with negative PCR results. Informed written consent was obtained from the study participants before sampling, and each participant was asked to answer a questionnaire form including demographic data such as age, gender, occupation, high-risk behaviors, and history of symptoms compatible with COVID-19 as of February 2020. The participants' occupations were divided into low-risk and high-risk jobs (like clinical and nonclinical hospital staff, taxi drivers, and jobs in crowded places like peddlers). Volunteer participants with a history of traveling, using public transportation, contact with symptomatic patients, or working at high-risk places were considered as high-risk behavior volunteers. Patients with a history of symptoms compatible with COVID-19 as of February 2020 were divided into four groups based on their clinical signs: asymptomatic, mildly symptomatic

(fatigue, runny nose, sneezing, nausea, and diarrhea), symptomatic (fever, cough, shortness of breath), and severely symptomatic patients requiring hospitalization. Sera were separated immediately and stored at -20 °C until used in the tests. Symptomatic volunteers at the sampling time were separated from other volunteers.

Ethical statements: The Ethics Committee of Tarbiat Modares University (IR.MODARES.REC 1399.009) and Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1399.299) approved the study.

Serological assessment: Detection of SARS-COV-2 antibodies was done with an ELISA kit targeting N or S protein (Pishtazteb-Iran) according to the manufacturer's protocol. Anti-N IgG SARS-COV-2 antibody ELISA kit with 94.1% sensitivity and 98.3% specificity and anti-S IgG ELISA kit with 85.3% sensitivity and 99.01 % specificity were used for samples of volunteer participants. IgM SARS-COV-2 antibody ELISA kit coated with N and S proteins with 79.4% sensitivity and 97.3% specificity was used for samples of hospitalized patients with negative PCR results. Optical densities greater than the cut-off value were considered as positive.

Statistical analysis: The prevalence rates of anti-N IgG, anti-S IgG, and anti-N and anti-S IgM seropositivity were estimated with 95% confidence intervals (CIs) and adjusted for clustering by location through cluster robust standard errors [9-12]. The prevalence estimates were adjusted for test performance (i.e., sensitivity: Se, and specificity: Sp) using a Monte Carlo bias analysis with 100,000 samples based on the following formula: $TP = (AP + Sp - 1) / (Se + Sp - 1)$, where TP denotes true prevalence, and AP denotes apparent prevalence [13, 14]. Beta distribution was used for sensitivity and specificity, α was set as the number of true positives, and β was set as the number of false negatives as reported by the manufacturer (Pishtaz-

teb-Iran) so that the mean and variance of beta distribution were approximately equal to the mean and variance of sensitivity or specificity estimates. Apparent prevalence was drawn from a normal distribution with mean and standard deviation equal to the prevalence estimate and its standard error, respectively. Point estimates and 95% simulation intervals (called confidence intervals in this paper for simplicity) were derived using the median and 2.5th and 97.5th percentiles of Monte Carlo distribution [15]. Multiple logistic regression was used to obtain adjusted odds ratios (ORs) between variables and anti-N IgG seropositivity with 95% CIs [16, 17]. Paired t-test was used to compare anti-N and anti-S antibody titers on a logarithmic scale, and the results were presented as geometric mean ratio with 95% CI. All analyses were performed in Stata version 14 (Stata, <https://www.stata.com/>).

Findings

Totally, 797 serum samples were collected. Of which 716 samples were from asymptomatic or symptomatic volunteer participants who did not seek medical care because of COVID-19 symptoms, and 81 samples were from symptomatic hospitalized patients whose SARS-COV-2 molecular test results were negative. The median age was 37.4 years (ranging from 5 to 92 years) in volunteer participants and 56.07 years (ranging from 20 to 92 years) in symptomatic hospitalized patients. In terms of gender distribution, 50.7 and 50% of volunteer participants and symptomatic hospitalized patients were female, respectively. Almost 50% of volunteer participants had high-risk jobs, of which 39% were clinical or non-clinical hospital staff. The behavior of 70.8% of volunteer participants was considered as high-risk. The characteristics of the samples are summarized in Table 1.

The prevalence rates of SARS-COV-2 anti-N

Table 1) Characteristics of volunteer participants and hospitalized patients with negative PCR results

Characteristic		Volunteer Participants (%)	Hospitalized Patients (%)
Gender	Male	49.3	50
	Female	50.7	50
Age (year)	<15	9.2	0
	16-59	84.0	55.9
	>60	6.8	44.1
Occupation	Low risk	49.7	Indeterminate
	High risk	50.3	Indeterminate
Behavior	Low risk	29.2	Indeterminate
	High risk	70.8	Indeterminate
Symptom	Asymptomatic	70.5	0
	Mildly symptomatic	18	0
	Symptomatic	11.5	0
	Severely symptomatic	0	100
Anti -N Ab	Negative	82.3	77.8
	Positive	17.7	22.2

IgG and anti-N/S IgM antibodies (95% CI) in volunteer participants and hospitalized patients with negative SARS-COV-2 PCR were 17.7% (10.4-25.1%) and 22.2% (13.1-31.3%), respectively. After adjustment for test performance, these values were 17.3% (8.8-25.8%) and 25.5% (12.8-39.7%), respectively. About 70% of volunteer participants had no symptoms related to SARS-COV-2, while 13.7% of them had positive COVID-19 antibody results. People between 15 and 60 years of age (compared to those under 15), men, as well as those with high risk occupations (hospital workers and taxi drivers), high risk behaviors, and symptomatic diseases were at higher risk of anti-N SARS-COV-2 IgG antibody positivity (Table 2). SARS-COV-2 anti-S antibody was evaluated in volunteer participants who were positive for SARS-COV-2 anti-N antibody (Fig. 1). Among

anti-N antibody positive subjects, the percentage of anti-S antibody positive individuals was 42.5% (19.3 and 65.7%); the performance-adjusted prevalence was 49.2% (21.4 and 78.8%). In other words, the results showed that in 50.8% (21.2 and 78.6%) of SARS-COV-2 anti-N antibody positive volunteer participants, the anti-S antibody, which is more correlated with neutralizing antibodies, was negative. The analysis of anti-N and anti-S antibody titers (OD values) showed that the geometric mean value of anti-N titer was 3.86, while this value for anti-S titer was 0.72. The geometric mean ratio with 95% CI was 5.36 (4.25 and 6.75) ($p<.001$).

Discussion
This study showed the presence of anti-N antibody-positive cases among asymptomatic or mildly symptomatic SARS-COV-2 infected people as well as severely symptom-

Table 2) Adjusted associations between characteristics and COVID-19 Ab results in volunteer participants

Characteristic		COVID-19 Anti N IgG ⁻ (%)	COVID-19 Anti N IgG ⁺ (%)	OR* (95% CI)	P-Value
Gender	Female	83.6	16.4	-	
	Male	82.2	17.8	1.39 (1.2, 1.89)	.039
Age (year)	<15	84.1	15.9	-	
	16-59	81.7	18.3	1.30 (1.07, 1.56)	.007
	>60	87.2	12.8	1.19 (0.90, 1.57)	.23
Occupation	Low risk	88	12	-	< .001
	High risk	78	22	1.66 (1.57, 1.76)	
Behavior	Low risk	89.5	10.5	-	< .001
	High risk	80.5	19.5	1.29 (1.24, 1.35)	
Symptom	Asymptomatic	86.3	13.7	-	
	Mildly symptomatic	80.6	19.4	1.40 (0.73, 2.67)	.31
	Symptomatic	61	39	4.00 (2.25, 7.10)	< .001

*ORs are adjusted for other variables in the table

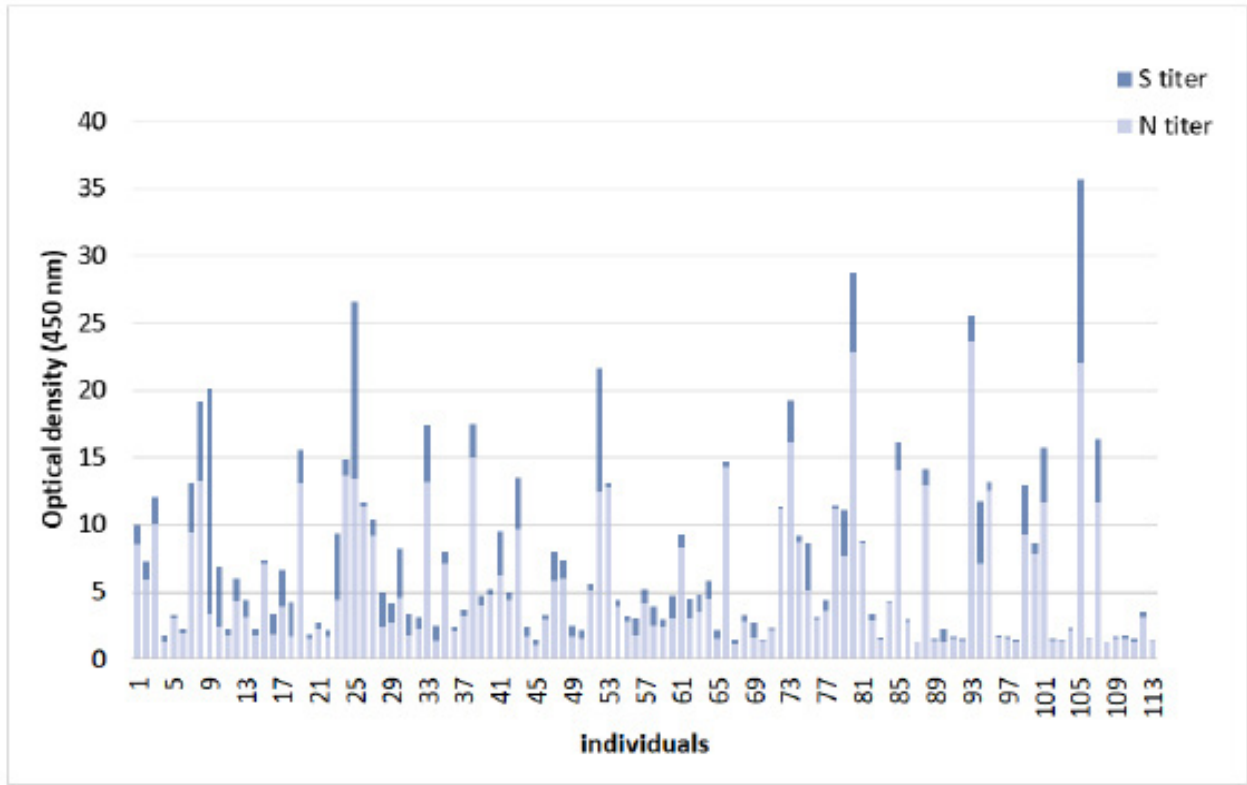


Figure 1) Comparing SARS-COV-2 anti-N and anti-S IgG levels (OD values) in seropositive volunteers

atic COVID-19 patients with negative PCR results, the obtained data are consistent with the reported seroprevalence in other regions of Iran in a relatively similar period of time^[10, 11]. This indicates that a number of people in the community are infected with the virus while they have no symptoms or confirmed PCR tests and may contribute to the virus spread^[7, 8]. These people could also play an important role as immune individuals in breaking the chain of virus transmission, but not all of them are protected against re-infection as infection with SARS-COV-2 is not always correlated with protection. These cases are detected based on the presence of anti-N antibody and may not be protected against the virus because they lack neutralizing antibodies. Anti-S antibody is partly correlated with neutralizing antibodies due to its RBD epitopes as the main region for interaction with cell receptors and production of neutralizing antibodies. The obtained data revealed that more than half of anti-N antibody positive people had negative anti-S antibody results; hence, they may not be protected against re-infection. Even assuming that the anti-S immune response in infected people is persistent for months and protective against re-infection, a herd immunity strategy is not easily achievable without mass vaccination^[3, 18, 19].

This study also showed that there is an association between positive SARS-COV-2 anti-N antibody results and high-risk behaviors such as traveling, using public transportation, contact with symptomatic patients, and working at high-risk places (like hospital staff, taxi drivers, and working in crowded places like peddlers). This finding is consistent with the finding of another study that showed a relationship between the diagnosis of asymptomatic COVID-19 patients and virus-contaminated environments^[20].

Consistent with a cross-sectional study that investigated SARS-COV-2 antibodies among

asymptomatic hospital staff^[21], this survey found an association between positive SARS-COV-2 anti-N antibody results and high-risk occupations. A high percentage of people with high-risk jobs in this study were clinical or non-clinical hospital staff. This means that better protection protocols must be implemented in medical centers to reduce the incidence of new cases^[22]. Since the circulation of the virus in the community could not provide the necessary immunity in individuals, by reducing the antibody titer, high-risk populations are again exposed to the risk of infection. Therefore, vaccination of high-risk groups and evaluation of the production and persistence of neutralizing antibodies after vaccination should be one of the priorities of the health system.

As expected, the data of this survey indicated the existence of false-negative results in molecular tests. Simultaneously performing molecular and serological COVID-19 tests leads to a decrease in false-negative molecular test results. Accordingly, this could help in timely isolation of COVID-19 patients and reduce the virus spread.

This study had several limitations such as the limited number of participants in each study group, which reduced the chance of randomized sampling. In addition, evaluating anti-RBD antibodies or measuring neutralizing antibodies using a virus neutralization test could provide a more accurate assessment of protection status in an unvaccinated community.

Conclusion

In conclusion, the obtained results revealed that there is the risk of virus spreading among cases without symptoms or confirmed molecular tests because protective antibodies may not be produced in these infected individuals. The results of this study reflect the antibody status of the population only at the time of sampling, and due to the

variability of recent epidemic conditions in the community, continuous studies in this field are necessary to clarify the situation and extent of the spread of COVID-19 in the society.

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Ethical statements: The Ethics Committee of Tarbiat Modares University (IR.MODARES.REC 1399.009) and Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1399.299) approved the study.

Conflicts of interests: There are no conflicts of interests for any author.

Authors' contributions: N Hajiahmadi as the main researcher along with F Mojtahedzade and A Yari performed sampling of volunteers in Tehran and laboratory tests. She also prepared the manuscript. M Tat and R Dorostkar coordinated and managed the hospital samples. H Soleimanjahi was the project consultant, and M Jafari, S Asli, and S Jamehdar performed sampling and testing in Mashhad. M Nazemipour was a statistical consultant, and M Mansournia was the designer and epidemiological consultant. T Bamdad was the project manager.

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Consent to participate: Written informed consents were obtained from all patients.

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