

Coronavirus Pandemic

Virus-specific humoral immune response in Bulgarian COVID-19 patients with varying disease severity

Evelina Shikova¹, Yuliya Marteva-Proevska^{1,2}, Tsanko Mondeshki^{3,4}, Radoslav Bilyukov^{3,4}, Daniela Marinova⁵, Elisaveta Naumova^{5,6}

¹ University Hospital "Alexandrovska", Central Microbiology Laboratory, Sofia, Bulgaria

² Medical University - Sofia, Medical Faculty, Department of Medical Microbiology, Sofia, Bulgaria

³ University Hospital "Alexandrovska", Clinic of Propedeutics of Internal Medicine, Sofia, Bulgaria

⁴ Medical University - Sofia, University Hospital "Alexandrovska", Department of Propedeutics of Internal Medicine, Sofia, Bulgaria

⁵ University Hospital "Alexandrovska", Clinic of Clinical Immunology and Stem Cell Bank, Sofia, Bulgaria

⁶ Medical University - Sofia, Medical Faculty, Department of Clinical Immunology, Sofia, Bulgaria

Abstract

Introduction: Our study aimed to analyze virus-specific humoral immune responses in COVID-19 patients with varying disease severity.

Methodology: A total of 109 serum samples from 87 patients, symptomatic for COVID-19 were studied using anti-SARS-CoV-2 immunoassays detecting different classes of immunoglobulins.

Results: Clinical samples were divided into 2 groups - collected up to and more than 2 weeks post-onset of symptoms (PoS). In the first group, the highest percentage of positive samples was found for IgA class virus-specific antibodies (78.1%), followed by IgM (71.9%/59.4%) and IgG (56.3%/53.1%). In the second group, samples positive for virus-specific IgA class antibodies were also the most (97.7%) along with those positive for IgG. A total of 72 IgA and/or IgM and/or IgG positive samples were further tested for SARS-CoV-2 neutralizing antibodies (NAbs) - 89.1% and 100% of samples obtained up to and after 2 weeks PoS, respectively were positive. Serological test results were also analyzed depending on the severity of the disease - SARS-CoV-2 positive samples in mild forms of COVID-19 were fewer than in moderate and severe forms but this difference was not statistically significant.

Conclusions: SARS-CoV-2 specific antibodies and a high virus neutralization capacity of these antibodies appear early PoS; Immunoglobulins of IgA class are of most significant diagnostic value for detection of SARS-CoV-2 infection; Timing of testing is the most important factor for positivity rate.

Key words: SARS-CoV-2; anti-SARS-CoV-2 antibodies; serological tests; timing of testing; disease severity.

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Introduction

Detection of SARS-CoV-2 RNA in respiratory tract samples is considered the gold standard for laboratory diagnosis of COVID-19. At the same time, since the beginning of the COVID-19 pandemic, several serological methods have been developed to detect antibodies against SARS-CoV-2, including enzyme-linked immunosorbent assay (ELISA), enzyme-linked fluorescence assay (ELFA), chemiluminescent immunoassay (CLIA), rapid immunochromatographic assay (RICA). These laboratory methods are designed for the detection of different classes of virus-specific immunoglobulins (IgM, IgA, and/or IgG) and different epitopes of SARS-CoV-2 (N/nucleocapsid and S/spike

protein). However, standardization of methods performance has not yet been achieved.

According to previous studies, antibody seroconversion in most patients infected with SARS-CoV-2 occurs 7 to 14 days post-onset of symptoms (PoS) [1]. Serological testing can identify individuals who have already developed virus-specific antibodies, thereby detecting past infections and providing better information about the spread of virus in population. Unlike viral RNA, virus-specific antibodies remain in the blood for several weeks to months or years PoS. Since many SARS-CoV-2 cases are asymptomatic, serological studies are particularly useful and due to the possibility of being carried out on a large scale to assess the overall immune response in the population [2].

As serological tests alone may not be sufficient to diagnose SARS-CoV-2, combining serological and molecular techniques could improve diagnostic accuracy. Asymptomatic individuals as well as those with suspected infection but negative for SARS-CoV-2 RNA can be diagnosed using virus-specific serological tests.

At the same time, elements of immune response in SARS-CoV-2-infected patients remain unclear. For example, it is not clear, to what extent the characteristics of seroconversion in the virus-specific immune response can be used as biomarkers for disease prognosis and the application of adequate therapeutic strategies. Answers to these questions will also contribute to the improvement of SARS-CoV-2-infection control and will make us better prepared for future challenges with this virus.

In this regard, many groups, including ours, have studied the role of antibodies in COVID-19 patients. In the present study, virus-specific humoral immune response was investigated in COVID-19 patients with varying disease severity using anti-SARS-CoV-2 immunoassays detecting different classes of immunoglobulins.

Methodology

Patients and clinical samples

A total of 109 serum samples obtained from 87 patients aged between 25 and 81 years (37 women and 50 men), symptomatic for COVID-19 and with PCR-proven SARS-CoV-2 infection, were studied. The patients had different severity of the course of the disease - mild ($n = 18$), moderate ($n = 38$), and severe ($n = 31$). The patients have been admitted to the Departments for COVID-19 treatment of University Hospitals “Alexandrovska” and “St. Anna” in Sofia during the period of April-November, 2020. All clinical samples were obtained with informed consent of the patients according to the national regulations and the ethical requirements of the hospital. 64 samples were taken in the first two weeks PoS, 43 - after 2 weeks PoS, 2 – period PoS unknown. For 22 of all patients 2 samples were tested for each person: first of them collected up to 2 weeks PoS and the other - after second-week PoS.

To study the development of a humoral immune response to SARS-CoV-2, tests were performed to detect specific antibodies from IgA, IgM, and IgG classes in the patients' serum samples. For the detection of antibodies from IgM and IgG classes, two different methods were used to compare their diagnostic performance. Subsequently, for patients with detected

specific antibodies of any of the indicated classes, additional tests were performed to determine the presence of virus neutralizing antibodies (NABs).

Serological tests to prove anti-SARS-CoV-2 antibodies

The presence of IgA antibodies against S1-antigen of SARS-CoV-2 was determined by ELISA. Tests were performed using a commercial kit -Anti-SARS-CoV-2 ELISA (IgA) (EI 2606-9601 A, EUROIMMUNAG/PerkinElmer, Germany). According to the manufacturer, the specificity of the test is 98.3%. The sensitivity depends on the time of sample collection (time point after onset of symptoms or positive pathogen detection) - 88.2% (≤ 10 days) and 96.9% (11 and 60 days).

The presence of IgG class antibodies against SARS-CoV-2 was determined using two methods - ELISA and RICA. Commercial kits, Anti-SARS-CoV-2 ELISA (IgG) (anti S1 protein) (EI 2606-9601G, EUROIMMUNAG/PerkinElmer, Germany) and Rapid SARS-CoV-2 Antibody (IgM/IgG) test (Advanced Quality, InTecProducts, INC, China, REF ITP 16001-TC-25) were used, respectively. According to the manufacturers, the specificity and the sensitivity of these tests are as follows: Anti-SARS-CoV-2 ELISA (IgG) has specificity of 99.6% and the sensitivity depends on the time of sample collection (time point after onset of symptoms or positive pathogen detection) - 43.7% (≤ 10 days) and 94.4% (> 10 days); Rapid SARS-CoV-2 Antibody (IgM/IgG) has specificity and sensitivity of 99.07% and 96.02%, respectively. The presence of IgM class antibodies against SARS-CoV-2 was determined using two methods - ELISA and RICA. Commercial kits -VIDAS SARS-COV-2 IgM (9COM) (anti S1 protein) (REF 423833-01, Biomérieux, France) and Rapid SARS-CoV-2 Antibody (IgM/IgG) test (Advanced Quality, InTecProducts, INC, China, REF ITP 16001-TC-25) were used, respectively. According to the manufacturer, VIDAS SARS-COV-2 IgM has a specificity of 99.6%, the sensitivity depends on time of sample collection (time point after positive PCR result) - 52.9% (≤ 7 days), 90.6% (8 - 15 days) and 100% (≥ 16 days).

The presence of Nabs against the receptor binding domain (RBD) of the S-antigen was determined by blocking enzyme-linked immunosorbent assay, performed with a commercial cPass SARS-COV-2 Neutralization Antibody detection kit (Nanjing GenScript Biothec Co, Ltd, China). According to the manufacturer, the test has specificity of 96.7%, Positive and Negative Percent Agreement with the Plaque Reduction Neutralization Test of 100%.

Table 1. Summary of serological test results.

Test	Time from the symptoms onset	Number (N)	Positive (N, %)	Negative (N, %)	borderline (N, %)
ELISA SARS-CoV-2 IgA	< 2 weeks	64	50 (78.1%)	11 (17.2%)	3 (4.7%)
	> 2 weeks	43	42 (97.7%)	0	1 (2.3%)
ELISA SARS-CoV-2 IgG	< 2 weeks	64	34 (53.1%)	25 (39.1%)	5 (7.8%)
	> 2 weeks	43	42 (97.7%)	1 (2.3%)	0
Rapid SARS-CoV-2 - IgM	< 2 weeks	64	46 (71.9%)	18 (28.1%)	NA
	> 2 weeks	34	31 (91.2%)	3 (8.8%)	NA
Rapid SARS-CoV-2 - IgG	< 2 weeks	64	36 (56.3%)	28 (43.8%)	NA
	> 2 weeks	34	32 (94.1%)	2 (5.9%)	NA
VIDAS ELFA SARS-CoV-2 IgM	< 2 weeks	64	38 (59.4%)	26 (40.6%)	NA
	> 2 weeks	32	29 (90.6%)	3 (9.4%)	NA
cPass SARS-CoV-2 Neutralization Antibody detection kit	< 2 weeks	46	41 (89.1%)	5 (10.9%)	NA
	> 2 weeks	26	26 (100%)	0	NA

All tests were performed in accordance with manufacturers' instructions.

Statistical analysis

Categorical variables are presented as number and percentage. The relationship between disease severity and categorical variables was evaluated by the Chi-square test or Fisher's exact. Data analysis was performed with the help of Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp., Armonk, NY, USA). A *p* value < 0.05 was considered statistically significant.

Results

Virus-specific antibodies were detected even 1st day PoS (1 patient of 2 tested). In the group of patients with serum samples obtained up to 2 weeks PoS, the highest percentage of positive samples was found for IgA class virus-specific antibodies (78.1%), followed by IgM (71.9% and 59.4%, respectively with RICA and ELFA) and IgG (56.3% and 53.1% with RICA and ELISA, respectively) (Table 1). In the group of samples collected after 2 weeks PoS, samples positive for virus-specific IgA class antibodies were also the most (97.7%) along with those positive for IgG class antibodies (97.7% and 94.1% by ELISA and RICA). In this period, samples positive for IgM class antibodies were the least (91.2% and 90.6% with RICA and ELFA,

respectively). A total of 72 IgA and/or IgM and/or IgG positive samples were further tested for SARS-CoV-2 NAbs - 46 obtained up to 2 weeks PoS, and 26 – after 2 weeks PoS. 89.1% of samples of the first group and 100.0% of those of the second group were positive.

The percentage of positive samples for all types of tests was higher in patients tested after the second week of PoS compared to that of patients tested in the first two weeks of PoS (Table 2). This percentage increased as follows: from 78.1% to 97.7% for ELISA SARS-CoV-2 IgA, from 53.1% to 97.7% for ELISA SARS-CoV-2 IgG, from 71.9% to 91.2% for Rapid SARS-CoV-2 IgM, from 56.3% to 94.1% for Rapid SARS-CoV-2 IgG, from 59.4% to 90.6% for VIDAS ELFA SARS-CoV-2 IgM and from 89.1% to 100.0% for SARS-CoV-2 NAbs detection. In most types of serological tests, these differences were statistically significant (*p* ranged between 0.00001 and 0.0373). An exception is the data on SARS-CoV-2 NAbs detection, where differences were not statistically significant (*p* = 0.1518).

For twenty-two of all patients included in the study two samples were tested for each person - first collected up to two weeks PoS and the second -after two weeks PoS. The first serum samples were negative (for all classes virus specific antibodies) in 4 of these patients and positive (most often antibodies from class IgA were detected) in the remaining 18 patients. Second samples

Table 2. Positivity of serological samples according to the time since the onset of COVID-19 symptoms.

Test	Positive samples		<i>p</i>
	Obtained up to two weeks from the symptoms onset N (%)	Obtained after two weeks from the symptoms onset N (%)	
ELISA SARS-CoV-2-Ratio-IgA (n/p)	50/64 (78.1%)	42/43 (97.7%)	0.0039
ELISA SARS-CoV-2-Ratio-IgG (n/p)	34/64 (53.1%)	42/43 (97.7%)	0.00001
Rapid SARS-CoV-2 Antibody (IgM/IgG) Test IgM (n/p)	46/64 (71.9%)	31/34 (91.2%)	0.0373
Rapid SARS-CoV-2 Antibody (IgM/IgG) Test IgG (n/p)	36/64 (56.3%)	32/34 (94.1%)	0.0001
VIDAS ELFA IgM SARS-CoV-2-TV (n/p)	38/64 (59.4%)	29/32 (90.6%)	0.0019
cPass SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript)-Inhibition % (n/p)	41/46 (89.1%)	26/26 (100.0%)	0.1518

Table 3. Summary of serological tests results for 22 patients with two samples.

Test	Time from the symptoms onset	Number (N) of tested samples	Results		
			Positive (N, %)	Negative (N, %)	borderline (N, %)
ELISA SARS-CoV-2 IgA	< 2 weeks	22	18 (81.8%)	3 (13.6%)	1 (4.5%)
	> 2 weeks	22	22 (100%)	0	0
ELISA SARS-CoV-2 IgG	< 2 weeks	22	12 (54.5%)	8 (36.4%)	2 (9.1%)
	> 2 weeks	22	22 (100%)	0	0
Rapid SARS-CoV-2 IgM	< 2 weeks	22	15 (68.2%)	7 (31.8%)	NA
	> 2 weeks	13	13 (100%)	0	NA
Rapid SARS-CoV-2 IgG	< 2 weeks	22	12 (54.5%)	10 (45.5%)	NA
	> 2 weeks	13	13 (100%)	0	NA
VIDAS ELFA SARS-CoV-2 IgM	< 2 weeks	22	13 (59.1%)	9 (40.9%)	NA
	> 2 weeks	11	11 (100%)	0	NA
cPass SARS-CoV-2 Neutralization Antibody detection kit	< 2 weeks	14	14 (100%)	0	NA
	> 2 weeks	7	7 (100%)	0	NA

of all patients of this group were positive for all classes of virus-specific antibodies. The results are shown in Table 3.

Serological test results were also analyzed depending on the severity of the disease (Table 4). For all test types SARS-CoV-2 positive samples in mild forms of COVID-19 were fewer than in moderate forms. The highest percentage of positive samples was observed in the group of patients with severe disease. This correlation was observed both in samples obtained up to 2 weeks and after 2 weeks PoS. An exception was observed in samples tested with ELISA SARS-CoV-2 IgG (up to 2 weeks PoS), where percentage of positive samples in the group with severe disease (52.2%) was lower than that in the group with moderate forms (58.1%). All samples collected after 2 weeks PoS from patients of both groups, with moderate and severe course of disease, were positive. Despite differences in positivity rates in the studied patient groups, in most cases, this difference was not statistically significant. Statistically significant differences in positivity rates according to the severity of disease were found for serum samples collected more than 2-week PoS and tested using Rapid SARS-CoV-2 Antibody (IgM/IgG) Test IgM ($p = 0.014$) and with VIDAS ELFA IgM SARS-CoV-2-TV ($p = 0.004$).

For testing of clinical samples for virus-specific immunoglobulins of class IgM and IgG we used 2

different methods to compare their diagnostic performance – ELFA and RICA to detect IgM; ELISA and RICA for detection of IgG. A complete concordance of results was found in 88.5% of samples tested for IgM and in 90.4% tested for IgG.

Discussion

In this study, we analyzed humoral virus-specific immune response in 87 patients with RT-PCR-proven SARS-CoV-2 infection and with varying disease severity. Serological tests for detection of IgA, IgM, IgG class immunoglobulins, and NABs were performed using tests based on different methods: ELISA, ELFA, rapid immunochromatographic analysis.

Our studies showed a high positive detection rate of all or individual specific immunoglobulin classes against SARS-CoV-2 as well as NABs in early stages PoS - 78.1% in samples collected up to 2 weeks PoS. Positive samples for virus-specific antibodies were found even on the 1st day PoS. These results are similar to those obtained by other authors in previous studies. According to the literature, antibody seroconversion in most patients infected with SARS-CoV-2 occurs 7 to 14 days PoS, but variation exists between individuals [1,3,4].

We also studied the correlation between the humoral immune response to SARS-CoV-2 and the period after the onset of COVID-19 symptoms by

Table 4. Distribution of positive serological samples according to the severity of the disease.

Time	Time from the symptoms onset	Positive samples			p
		Mild N (%)	Moderate N (%)	Severe N (%)	
ELISA SARS-CoV-2-Ratio-IgA (n/p)	< 2 weeks	5/10 (50.0%)	24/31 (77.4%)	21/23 (91.3%)	0.108
	> 2 weeks	8/9 (88.9%)	20/20 (100.0%)	14/14 (100.0%)	0.209
ELISA SARS-CoV-2-Ratio-IgG (n/p)	< 2 weeks	4/10 (40.0%)	18/31 (58.1%)	12/23 (52.2%)	0.555
	> 2 weeks	8/9 (88.9%)	20/20 (100.0%)	14/14 (100.0%)	0.209
Rapid SARS-CoV-2 Antibody (IgM/IgG) Test IgM (n/p)	< 2 weeks	5/10 (50.0%)	23/31 (74.2%)	18/23 (78.3%)	0.233
	> 2 weeks	6/9 (66.7%)	13/13 (100.0%)	12/12 (100.0%)	0.014
Rapid SARS-CoV-2 Antibody (IgM/IgG) Test IgG (n/p)	< 2 weeks	5/10 (50.0%)	17/31 (54.8%)	14/23 (60.9%)	0.826
	> 2 weeks	7/9 (77.8%)	13/13 (100.0%)	12/12 (100.0%)	0.064

dividing clinical samples into 2 groups (collected up to and more than 2 weeks PoS). In this analysis, we found an increase in positivity rate from 53.1-78.1% to 90.6-97.7% for samples collected before and after 2 weeks PoS, respectively, and for NAbS from 89.1% to 100%. Our studies showed statistically significant differences between number of positive samples taken up to 2 and after 2 weeks PoS with P varying between 0.00001 and 0.0373. An exception is a study for NAbS, where the differences are not statistically significant ($p = 0.1518$), but in this case, only samples positive for IgA and/or IgM and/or IgG virus-specific antibodies were examined. In fact, according to our results, the time PoS when the serological samples are collected is the most important factor for sample positivity. High positivity rates for samples collected at later stage PoS are also reported by other authors [3,5].

Conflicting data are described in the literature regarding the chronology of the appearance of IgM and IgG after infection with SARS-CoV-2, which can also be explained by the different sensitivity of the particular tests used in the study. According to some authors, dynamics in the production of antibodies against SARS-CoV-2 is typical of an acute viral infection with an increase in IgG, appearing with a decrease in IgM [6-9]. According to others, there is an earlier conversion for IgG than for IgM [10,11]. These conflicting data support the possibility of detecting both classes of virus-specific antibodies simultaneously [12]. In our case, the difference between IgM and IgG positivity rates before and after 2 weeks PoS is not significant and rather supports the idea of a possible co-detection/appearance of these antibodies.

In this study, a high percentage of samples positive for virus-specific IgA class antibodies is observed -this was found both for samples collected up to 2 weeks PoS (78.1%) and for those from a later period (97.7%). Compared to IgM and IgG class antibodies, this is the highest positivity rate. So, in our opinion, testing for immunoglobulins of IgA class is of significant diagnostic value for the detection of SARS-CoV-2 infection, especially in cases with negative RT-PCR but with suspicious clinical symptoms. Of course, there is also the hypothesis that the lower percentage of positive samples for IgM and IgG found in our case could also be due to the insufficient sensitivity of the specific tests used. Another possibility is that IgA may be more broadly cross-reactive against various human coronaviruses and IgA class antibody detection was not with high specificity (although the particular kit we used is based on S1-antigen of SARS-CoV-2, known to produce the highest specificity). A similar hypothesis is

found in the literature [8,13]. An early appearance of immunoglobulins of IgA class has also been observed in previous studies [6,14-18]. It was reported that IgA was more potent than IgG in neutralizing SARS-CoV-2 [16]. IgA is known to play a critical role in the defense of mucosal surfaces against pathogens by neutralizing respiratory viruses and preventing their attachment to epithelial cells. IgA-mediated mucosal immunity may be an important protective mechanism against SARS-CoV-2 and can reduce the infectivity of human secretions and viral transmission.

NAbS are critical for virus elimination and protection against SARS-CoV-2 [19,20]. SARS-CoV-2 NAbS are believed to be the critical indicator for evaluating clinical outcomes and vaccination effects. Most SARS-CoV-2 infected patients are positive for NAbS 14-20 days PoS [21]. Almost all patients develop NAbS by week 4 of infection, and severely ill patients show faster onset and higher titers of NAbS than mild cases [22,23]. Ren *et al.* found that NAbS appear in a time frame similar to other virus-specific antibodies [24]. Regarding the high percentage of positive samples for NAbS in our study both in earlier stages PoS (89.1%) and after 2 weeks PoS (100.0%), it should be noted that only positive samples for virus-specific IgA and/or IgM and/or IgG were further investigated with this test. This could also explain the lack of statistical significance of the difference in positive samples depending on PoS time.

In addition, to clarify kinetics in virus-specific humoral immune response we also analyzed the data from serological testing of the 22 patients group where 2 samples were tested for each person –up to and after 2 weeks PoS. The results showed similar dynamics of virus-specific antibody response and confirmed our initial conclusions.

We also investigated the correlation between disease severity and virus-specific antibody positivity rate. There are quite contradicting data on this issue in the literature. Several publications have strongly argued that there is a correlation between disease severity in patients with COVID-19 and humoral immune response against SARS-CoV-2 [8,23,25-28]. However, other authors have opposite opinion - that no such correlation exists [3,11]. A prognostic relationship between antibody kinetics and disease severity in COVID-19 has been reported. Thus, some authors found a delayed specific humoral immune response in patients with a severe course of COVID-19, in contrast to an earlier response in patients with a mild course [24,29,30]. According to others, seroconversion occurs earlier and antibody titers are higher in severe cases [9,25,31,32].

The results of our study showed an increase in positivity rates for virus-specific antibodies with increasing disease severity, and in most cases, this difference was not statistically significant. We found a statistically significant difference only for the IgM class antibodies examined in samples taken after more than 2 weeks PoS. Similar results were also reported in some previous publications, according to them IgM titer was significantly lower in patients with mild course than that of patients with severe course, but both groups showed a comparable immune response for IgG class antibodies [33]. Many studies have found a correlation between the level of virus-specific IgA antibodies and disease severity, with IgA antibody levels being higher in patients with more severe disease [15,18,27,28,34]. The fact that IgA levels correlate with disease severity may be due to a higher immune response of the respiratory system facing a severe lower respiratory tract infection, or that IgA has a pathogenetic role in the development of severe disease. We observed an increase of IgA-positive samples in patients with severe disease course, but this difference was not statistically significant ($p = 0.108$ and $p = 0.209$).

Our study has some limitations: Only 87 patients were included in the study leading to the small size of different patient groups; The serum sampling time (days post-onset of symptoms), although in time frame of up to or more than 2 weeks, was different for each patient; The size of patient group (22 persons) where 2 samples were tested for each person is insufficient for statistical analysis; Although the commercial kits used in this study are with high specificity, possible antibody cross-reactivity should be considered as well.

Conclusions

Our study provides additional data regarding humoral immune response against SARS-CoV-2 infection that could be useful in diagnosis and control of COVID-19: Anti-SARS-CoV-2 antibodies detected in this study, appear early after the onset of COVID-19 symptoms; Immunoglobulins of IgA class are of most significant diagnostic value for detection of SARS-CoV-2 infection—they appear early, persist for a long time and are detected in greatest number of clinical samples; Most of the samples positive for IgA, IgM, IgG were also positive for NABs, indicating a high virus neutralization capacity; Timing of testing is the most important factor for positivity rate; Severity of disease is, but not such a significant factor, for positivity rate of virus-specific antibodies; Results of testing clinical samples by two methods are comparable and provide an

opportunity for effective use of rapid immunochromatographic tests.

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Authors' Contributions

E.S. and Y.M-P: conception, methodology, laboratory work, data analysis, article writing, review and editing; T.M. and R.B.: sample collection and clinical evaluation; D.M.: statistical analysis; E.N.: conception, data analysis, review and editing, supervision.

All authors contributed to data analysis and final approval of the article.

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Corresponding author

Yuliya Marteva-Proevska, MD, PhD
 University Hospital “Alexandrovska”,
 Central Microbiology Laboratory,
 1431 Sofia, Bulgaria
 Tel: +359 2 92 30 436
 Email: proevska@abv.bg

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