Anti-phospholipids antibodies and immune complexes in COVID-19 patients: a putative role in disease course for anti-annexin-V antibodies

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Abstract

Introduction: Besides distinctive respiratory and digestive hallmarks, COVID-19 has been recently associated with a high prevalence of pro-inflammatory and hypercoagulable states known as “COVID-19 Associated Coagulopathy” (CAC), corresponding to a worsening in patients’ conditions, whose causes are still to be elucidated. A link between anti-phospholipids antibodies (aPLs) and viral infections has long been suggested. APLs are assessed for Anti-phospholipid Syndrome (APS) diagnosis, characterized by thrombocytopenia, thrombosis and coagulopathy. Furthermore, circulating immune complexes (CICs), arisen upon inflammatory responses and related immune dysregulation, can lead to endothelial cells damage and thrombotic complications.

Method: We performed an extended panel including IgG/IgM anti-cardiolipin, IgG/IgM anti- β 2-glycoprotein-1, coupled with IgG/IgM anti-prothrombin, IgG/IgM anti-annexin-V on two COVID-19 patient groups (early and late infection time) and a negative control group. IgG CICs analysis followed to evaluate inflammatory status, through a possible complement system activation.

Results: Our results showed low positive cases percentage in IgG/IgM anti-cardiolipin and IgG/IgM anti- β 2-glycoprotein-1 assays (4.54%, 6.25%, 4.55%; in early infection group, late infection group and control group, respectively); few positive cases in IgG/IgM anti-prothrombin and IgG/IgM anti-annexin-V immunoassays; no IgG CICs positivity in any patient.

Conclusions: In conclusion, our data show a low aPLs prevalence, likely excluding an involvement in the pathogenesis of CAC.

Interestingly, IgG/IgM anti-prothrombin and anti-annexin-V positive cases, detected in late infection group, suggest that aPLs could temporarily increase or could trigger a “COVID-19-induced-APS-like-syndrome” in predisposed patients.

Finally, even though aPLs are transient, they may still have a thrombotic potential in genetically predisposed COVID-19 patients.

Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a new β -coronavirus strain identified for the first time in Wuhan, China, on late December 2019. On February 2020, the World Health Organization (WHO) named the infected disease “coronavirus disease 2019” (COVID-19) [1].

SARS-CoV-2 primarily spread from person to person via mucosae or conjunctiva after close contact with infected droplets, and respiratory and gastrointestinal systems epithelial cells are the main infection targets. First observations on disease onset resulted in progressive respiratory failure owing to alveolar damage, and classical symptoms included fever, cough, sore throat, malaise and myalgias. Nevertheless,

later on, many reports also described gastrointestinal symptoms, including anorexia, nausea and diarrhea [2]. Recently, evidences of cardiovascular and cerebrovascular systems involvement have been shown [3].

In a second time, in COVID-19 a high prevalence of coagulation abnormalities and thrombotic complications has been found, such as: Deep Vein Thrombosis (DVT), Pulmonary Embolism (PE), Venous Thromboembolism (VTE) and Disseminated Intravascular Coagulation (DIC) [4-5].

These clinical manifestations have been called “COVID-19 Associated Coagulopathy” (CAC), corresponding to a general worsening of patient conditions. Indeed, presence of small and mid-sized pulmonary arteries thrombosis and microangiopathy was found in COVID-19 patients, due to markedly low levels of oxygen. Subsequent respiratory failure causes Intensive Care Unit (ICU) admission and mechanical ventilation [6-8].

CAC is characterized by increased levels of routine clinical inflammation markers such as C Reactive Protein (CRP), Erythrocyte Sedimentation Rate (ESR), together with acute inflammatory response (cytokine storm): Interleukin-6 (IL-6), Interleukin-1 (IL-1) and Tumor Necrosis Factor α (TNF α). IL-6 levels are significantly higher in patients with severe conditions [9-10].

Regarding clinical laboratory data, coagulation parameters have been found altered in these patients [9,11], such as: activated partial thromboplastin time (aPTT), antithrombin (AT) and prothrombin time (PT). Many reports evidenced increased D-dimer and fibrinogen levels, involved in the development of thrombotic complications and in poor prognosis [12-13].

The real cause of COVID-19 patients' coagulopathy has not yet been identified.

We can hypothesize three different pathological ways in CAC pathogenesis: I) SARS-CoV-2 infection in endothelial cells may cause loss of endothelial homeostasis and its physiological anticoagulant activity; II) the systemic inflammatory response and cytokine storm may increase atherosclerotic plaques rupture probability, in patients with previous cardiovascular events history; III) anti-phospholipids antibodies (aPLs) along with the development of circulating immune complexes (CICs) could be involved in thrombosis events.

We focused our study on the last hypothesis.

Anti-phospholipids antibodies positivity clinical manifestations include thrombosis, thrombocytopenia, coagulopathy and pregnancy complications with recurrent spontaneous abortions.

Furthermore, anti-phospholipids antibodies are essential clinical criteria in the Anti-phospholipid Syndrome (APS) diagnosis, a systemic autoimmune disease in which specific laboratory markers, such as: lupus anticoagulant (LAC), IgG and/or IgM anti-cardiolipin (aCL) and IgG and/or IgM anti- β 2-glycoprotein-1 (a β 2GP1) antibodies are crucial to the diagnosis. These antibodies in fact represent the most frequent aPLs. IgG/IgM anti-prothrombin (aPT) and IgG/IgM anti-annexin-V are also detected in a minority of cases, especially in clinical APS patients with negative classical anti-phospholipids

antibodies. In healthy population, positivity to anti-phospholipids antibodies has been found in about 5% of cases, with an uncertain relationship for increased risk to develop thrombotic events and APS. Complement activation is also required for the full APS clinical manifestation.

In addition, inflammatory responses and related immune dysregulation could trigger the development of circulating immune complexes (CICs), which leads to endothelial cells damage and organ inflammation through their tissue depositing and through complement system activation (C1q, C3), resulting in thrombotic complications. Moreover, macrophages could phagocytose CICs causing a hyperinflammatory response, typical in COVID-19 patients [14].

Preliminary evidences of a possible correlation between anti-phospholipids antibodies and coagulopathy in COVID-19 patients derives from Zhang et al., who reported 3 cases with thrombosis, aCL and a β 2GP1 positivity only for IgA class [15]. It should be noticed that lack of IgG and/or IgM aCL and a β 2GP1 precludes the possible role of anti-phospholipids antibodies in CAC and furthermore, the analyzed patients had a history of cardiovascular disease episodes, which increased itself the risk of subsequent thrombosis events.

Based on these observations, Harzallah et al. tested LAC on 56 patients, IgG/IgM aCL and a β 2GP1 on 50 patients. LAC positivity was found in 25 patients (45%), whereas IgG and/or IgM aCL and a β 2GP1 were detected only in 5 patients (10%), three of them associated to LAC positivity [16]. Some authors however highlight that high levels of CRP interfere with LAC detection methods [17].

In another study, Valle et al. reported a prospective observational study in 24 COVID-19 patients, who developed pneumonia and/or venous thromboembolism: 11 patients with PE (45.8%), 9 patients with DVT (37.5%) and 4 patients with VTE (16.6%); also in this cohort only 2 patients (8.3%) showed anti-phospholipids antibodies positivity (IgM aCL and IgM a β 2GP1), suggesting that anti-phospholipids antibodies might not be involved in COVID-19 coagulopathy and thrombosis mechanism [18].

The aPLs low prevalence was also reported by Borghi et al. on 122 COVID-19 patients [19]. In this cohort IgG/IgA/IgM a β 2GP1 were the most frequent antibodies in 15.6%, 6.6% and 9.0% of patients, respectively; IgG/IgM aCL were detected in 5.7% and 6.6% of patients. Moreover, they detected IgG and IgM anti-phosphatidylserine/prothrombin (aPS/aPT) antibodies in 2.5% and 9.8% of patients, respectively. No association between thrombosis and aPLs was found. Nevertheless, combination of the various aPLs criteria and antibody profiles could be useful to better characterize the risk assessment of thrombotic events in COVID-19 patients [20].

To our knowledge, anti-prothrombin (aPT), anti-annexin-V antibodies and CICs in COVID-19 patients have not been reported in the scientific literature.

In this perspective, to better assess the anti-phospholipids antibodies role in CAC, we performed a more extended study panel including classical aPLs (IgG/IgM anti-cardiolipin and IgG/IgM anti- β 2-glycoprotein-1), supported by other anti-phospholipids antibodies (IgG/IgM anti-prothrombin and IgG/IgM anti-

annexin-V). We also detected IgG human circulating immune complexes (CICs) to evaluate the inflammatory status, through a possible complement system activation. These antibodies were assessed on a COVID-19 patients' cohort compared to a control group (healthcare workers).

Patients And Methods

Serum samples were recovered in accordance with local ethical approvals (R.S.44.20), from "Tor Vergata" University COVID-Hospital of Rome hospitalized patients as follows: 44 positive RT-PCR-diagnosed SARS-CoV-2 patients (mean age 67.3 years $\pm$ 16.6 years; 25 males and 19 females), collected on days 1 to 9 from first access to Emergency Department and from first positive nasopharyngeal swab (early infection patients); 48 positive RT-PCR-diagnosed SARS-CoV-2 patients (mean age 69.7 years $\pm$ 13.3 years; 27 males and 21 females), collected on days 19 to 41 from first access to Emergency Department and from first positive nasopharyngeal swab (late infection patients) and 44 negative RT-PCR-diagnosed SARS-CoV-2 subjects (control group) (mean age 41.7 years $\pm$ 11.1 years; 23 males and 21 females) collected from "Tor Vergata" Hospital physicians and healthcare workers screened for internal surveillance. Patients admitted to respiratory system department were: 17/44 in early infection group and 13/48 in late infection group. All subjects of the groups had been tested by serological assays for IgM and IgG anti-SARS-CoV-2 antibodies detection (submitted data). Early and late infection groups are composed by different individuals.

Informed consent was obtained from all subjects enrolled in the study. Sera were separated by centrifugation at 2500 g for 10 minutes, within 1h from collection. The study was in accordance with the Helsinki Declaration, as revised in 2013.

Real Time-Polymerase Chain Reaction (RT-PCR)

Nasopharyngeal swabs were tested for SARS-CoV-2 infection with Seegene AllplexTM2019-nCoV Assay (Seegene, Seoul, South Korea), according to the manufacturer's protocols.

Automated RNA extraction and PCR setup were carried out using Seegene NIMBUS, a liquid handling workstation. RT-PCR was run on a CFX96TMDx platform (Bio-Rad Laboratories, Inc., CA, USA) and subsequently interpreted by Seegene's Viewer Software. The Seegene AllplexTM2019-nCoV Assay identifies the virus by multiplex real-time PCR targeting three viral genes (E, RdRP and N), thus complying with international validated testing protocols.

Chemiluminescence Immunoassay (CLIA)

Semi-quantitative determination of IgG/IgM aCL and IgG/IgM a β 2GP1 antibodies in human serum, was performed on the fully automated BIO-FLASH[®] instrument (Inova Diagnostics, San Diego, USA) with

QUANTA Flash® APS-aCL family and aβ2GP1 family reagents (Inova Diagnostics, San Diego, USA). The QUANTA Flash aCL and aβ2GP1 assays are chemiluminescent two-step immunoassays consisting of magnetic particles coated with cardiolipin and human purified β2GP1 proteins which capture, if present, aCL and aβ2GP1 anti-phospholipids antibodies from the sample. The emitted light is measured as relative light units (RLUs) by the BIO-FLASH optical system; RLUs are directly proportional to the aCL and aβ2GP1 concentration in samples: manufacturer's recommended cut-off >20 CU (Chemiluminescent Units).

Enzyme-linked immunosorbent assay (ELISA)

Immunoenzymatic assay "Prothrombin IgG/IgM ELISA kit" (Demeditec Diagnostics GmbH, Kiel, Germany), was performed for quantitative measurement of IgG and IgM autoantibodies against prothrombin proteins in human serum. Standards and diluted samples (1:100) were incubated for 30 minutes in wells coated with prothrombin antigens, allowing the binding to the specific IgG/IgM prothrombin antibodies. After washing, a conjugate solution labeled with horseradish peroxidase (HRP) was dispensed into each well for 15 minutes. Finally, a chromogenic solution containing HRP substrate (tetramethylbenzidine; TMB) was added for 15 minutes and the reaction was then stopped by an acidic solution. The absorbances were read spectrophotometrically at 450 nm on a Plate Reader (DAS srl, Rome, Italy). Optical densities are proportional to the quantity of specific IgG/IgM prothrombin antibodies present in the samples. The results were estimated from a calibration curve (0; 6.3; 12.5; 25; 50; 100 U/ml). IgG and IgM anti-prothrombin manufacturer's recommended cut-off values were >12 U/ml.

Immunoenzymatic assay "Eu-Annexin G/M" (Eurospital, Trieste, Italy) was performed for quantitative measurement of IgG and IgM antibodies against annexin-V in human serum. Standards and prediluted samples (1:100) were pipetted into wells precoated with purified annexin-V. After 30 minutes incubation at room temperature, the microwells contents was discarded and a conjugate solution labeled with horseradish peroxidase was dispensed for 15 minutes. At the end of incubation, TMB was added for 15 minutes and the reaction was then stopped by an acidic solution. The absorbances of the colorimetric reaction were read at 450 nm on a Plate Reader (DAS srl, Rome, Italy). Optical densities are proportional to the quantity of specific IgG/IgM annexin-V antibodies present in the samples. The results were calculated on the corresponding standard curve (0; 6.3; 12.5; 25; 50; 100 U/ml). IgG and IgM anti-annexin-V manufacturer's recommended cut-off values were >8 U/ml.

The immunoenzymatic assay "CIC-C1q ELISA (IgG)" (EUROIMMUN, Lubeck, Germany) was performed for quantitative determination of human circulating immune complexes, containing IgG antibodies directed against C1q protein. Standards and diluted samples (1:51) were incubated into microplate wells coated with C1q protein for 30 minutes. After washing, a conjugate solution labeled with horseradish peroxidase was dispensed into each well for 30 minutes. Finally, a chromogenic solution containing HRP substrate (tetramethylbenzidine; TMB) was added for 15 minutes and the reaction was then stopped by an acidic solution. The absorbances of the colorimetric reaction were read at 450 nm on a Plate Reader (DAS s.r.l.,

Rome, Italy) within 30 minutes and the results were calculated on the corresponding standard curve (2; 20; 200 RU/ml; RU=relative units). IgG CICs manufacturer's recommended cut-off value was >20 RU/ml.

Statistical Analysis

Results were calculated by Mann-Whitney test. More than two groups comparison was determined by non-parametric one-way ANOVA test (KRUSKAL-WALLIS test). Statistical significance was defined as $p < 0.05$. All data were analyzed using GraphPad Prism Software 8.4.3 (San Diego, California, USA). The investigators were blinded to the group allocation during the experiment.

Results

We analyzed a total of 92 SARS-CoV-2 positive patients and 44 negative controls (both confirmed by RT-PCR).

In all groups we first tested IgG/IgM aCL and IgG/IgM a β 2GP1 prevalence, performing a semi-quantitative automated chemiluminescent assay. In positive early infection group (n=44), 2 patients (4.54%) were positive to IgG/IgM aCL or IgG/IgM a β 2GP1. In particular: UPN3 (unknown patient number 3) had an IgG aCL concentration of 27,9 CU; UPN26 had an IgM aCL concentration of 34,3 CU and IgM a β 2GP1 concentration of 31,5 CU. In positive late infection group (n=48), 3 patients were positive (6.25%) to IgG/IgM aCL or IgG/IgM a β 2GP1. In particular: UPN7 had an IgG aCL concentration of 39.9 CU; UPN22 had an IgM a β 2GP1 concentration of 30.1 CU and UPN40 had an IgG aCL concentration of 31.9 CU. In negative control group (n=44), we detected 2 patients (4.55%) with anti-phospholipids antibodies positivity: UPN15 had an IgG aCL concentration of 56.7 CU and UPN27 had an IgM aCL and IgM a β 2GP1 concentration of 41.5 CU and 26.9 CU, respectively.

Next, we assessed IgG/IgM anti-prothrombin and IgG/IgM anti-annexin-V prevalence on the different groups with quantitative ELISA assays. Results are shown in Figure 1 and Figure 2, respectively.

The IgG and IgM anti-prothrombin median concentrations (Table 1) did not show a statistical significance among the groups ($p=0.1938$ and $p=0.3584$, respectively). No patient with IgG/IgM anti-prothrombin antibodies positivity was found in negative control group and in positive early infection group. In positive late infection group, 2 patients (4.16%) showed IgG prothrombin antibodies positivity: UPN3 (45.2 U/ml), UPN36 (28.5 U/ml) and 3 patients showed IgM prothrombin antibodies positivity: UPN14 (19.1 U/ml), UPN33 (20 U/ml) and UPN47 (18.5 U/ml; Figure 1).

IgG/IgM anti-annexin-V assay results (Table 1) showed statistically significant median concentrations among the groups ($p=0.0101$ and $p=0.0029$, respectively). IgG weak positivity (2.27%) was found in one patient, both in negative control group and early infection group: UPN16 (10.8 U/ml) and UPN3 (8.47 U/ml), respectively. In positive late infection group, 3 patients (6.25%) showed IgG anti-annexin-V antibodies positivity: UPN35 (23.75 U/ml), UPN37 (11.31 U/ml) and UPN42 (20.87 U/ml); moreover, 3

patients showed IgM anti-annexin-V antibodies positivity: UPN2 (16.25 U/ml), UPN22 (11.37 U/ml) and UPN23 (10.6 U/ml). This group showed statistically significant p values when compared separately to control group and early infection group, both for IgG ($p=0.0111$ and $p=0.0072$, respectively) and IgM class ($p=0.0072$ and $p=0.0019$, respectively; Figure 2). Finally, in each group there was a patient with a double aPLs positivity: UPN27 in negative control group, UPN26 in early infection group and UPN22 in late infection group.

At last, IgG CICs ELISA immunoassay was performed, and results are shown in Figure 3. Also in this case, a statistical significance among the groups has been found ($p=0.0008$). Median concentrations are similar between negative and late infection groups (1.045 RU/ml (range 0.1000-10.30 RU/ml) and 1.120 RU/ml (range 0.2900-9.660 RU/ml) respectively), whereas early infection group has a lower median concentration (0.66 RU/ml (range 0.1000-3.940 RU/ml); Table 1), leading to significant p values when it was compared to negative control group and late infection group ($p=0.001$ and $p=0.0008$, respectively). No IgG CICs positivity was found in any patient with the manufacturer's recommended cut-off value (>20 RU/ml).

Discussion

SARS-CoV-2 may predispose patients to pro-inflammatory and hypercoagulable states, and increased risk of thrombotic events and coagulation abnormalities named "COVID-19 Associated Coagulopathy". Pathophysiological mechanisms of CAC remain uncertain and are under intensive investigation [4-8].

Since aPLs have long been considered as one of the contributors to the hypercoagulable states with following thrombotic events, we decided to perform a study to evaluate a possible correlation between anti-phospholipids antibodies and thrombosis events in COVID-19 patients. In addition, anti-phospholipids antibodies are essential clinical criteria in the anti-phospholipid syndrome [21-25] and their association to viral infection has long been suggested [26].

In our data, we have first identified few positive IgG/IgM aCL and IgG/IgM $\alpha\beta 2\text{GP1}$ patients in all groups, according to manufacturer's recommended cut-off value (>20 CU).

As previous studies have shown that moderate to high titers of aPLs display better clinical significance [9], we then reanalyzed data with a more stringent cut-off value (>40 CU): aPLs positivity was confirmed only in 2 negative controls. Therefore, lack of uniformity and the low percentage of positive cases in IgG/IgM aCL and IgG/IgM $\alpha\beta 2\text{GP1}$ assays, preclude a possible role of aCL and $\alpha\beta 2\text{GP1}$ antibodies in our cohort.

Likewise, IgG/IgM anti-prothrombin and IgG/IgM anti-annexin-V immunoassays showed few positive cases. Interestingly, IgG/IgM anti-prothrombin and IgG/IgM anti-annexin-V data show that distribution of positive cases number increases in late infection patients, significantly in anti-annexin-V results, suggesting a possible role for these anti-phospholipids antibodies in disease course. In fact, it has been reported that aPLs can arise transiently in some patients with critical illness and SARS-CoV-2 infection

(disappearing in a few weeks) [22]; as well as in other genetically predisposed patients they could trigger a “COVID-19-induced-APS-like-syndrome” or other autoimmune diseases [27-28]. Unfortunately, we could not perform a longer-term follow up.

To note, LAC positivity was found only in two late infection group patients (UPN2 with IgM anti-prothrombin positivity and UPN14 with IgM anti-annexin-V positivity).

Patients with any aPLs positivity were not admitted to ICU, suggesting that presence of these antibodies is not associated to disease severity. Furthermore, D-dimer and fibrinogen levels were higher compared to healthy population even though their means were similar to aPLs negative patients (data not shown).

Regarding IgG CICs immunoassay, we have identified no positive cases in all patients' groups and negative control group. Since CICs are involved in inflammatory phenomena, we would have expected a significant increase, especially in positive early infection group. Conversely, IgG CICs median concentration compared to negative control group, decreased from 1.045 RU/ml to 0.66 RU/ml. Notably, in positive late infection group, IgG CICs median concentration increased to that of negative control group (1.120 RU/ml). These data could be explained with a possible CICs tissue precipitation in inflammation early phases and a subsequent restoring of the normal CICs concentration, in accordance to patients' clinical manifestations improvement. Nevertheless, our results suggest that in COVID-19, IgG CICs could not be considered as possible infection markers.

SARS-CoV-2 pandemic requires the identification of reliable and significant markers to quickly discriminate COVID-19 patients with general worsening of clinical conditions and increased risk of developing thrombotic events and coagulopathy abnormalities. Unfortunately, our results showing a low anti-phospholipids antibodies prevalence, pointed out that aPLs could not be considered as valid disease markers, considering that a higher clinically significant cut-off value, did not identify any positivity in the infection groups.

However, correlation between multifactorial clinical parameters, such as: activities of natural anticoagulants, coagulation factor levels, pro-inflammatory cytokines levels, D-dimer and fibrinogen levels, presence of anti-phospholipids antibodies and CICs, may contribute to develop an algorithm useful to the identification of critical ill COVID-19 patients [29].

Finally, despite IgA aCL and IgA a β 2GP1 are not included in the current APS classification criteria [30], recent evidences highlight a possible role for IgA anti-phospholipids antibodies in genetically predisposed patients [27]. Being IgA antibodies the most important immunoglobulins to counteract infectious pathogens in respiratory and digestive systems, they have an important role in mucosal immunity. In this perspective, a secondary anti-phospholipid syndrome should be considered in COVID-19 patients with positive aPLs.

Unfortunately, our work has several limitations. The study population has been chosen on the basis of available samples previously detected for anti-SARS-CoV-2 serological assays, therefore it includes

healthy subjects as a control group. It is well known that aPLs positivity is often associated to infectious diseases, thus a more informative comparison could have been between COVID-19 patients and those affected by other pneumological diseases. According to hospital data access policy, we can not provide any further information on medical records and on the clinical status of the patients, except for those results regarding laboratory medicine department.

In conclusion, our data show a low aPLs prevalence in accordance with previous studies, suggesting that these autoantibodies might not be involved in the pathogenesis of CAC, but they could arise transiently in COVID-19 patients.

These data could have a potential clinical implication in SARS-CoV-2 infection, proposing that even though autoantibodies are transient, they may still have a thrombotic potential in genetically predisposed COVID-19 patients. Long-term follow up and prospective evaluations of those aPLs should be performed to verify their persistence and pathogenicity.

Declarations

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Conflicts of interest: The authors declare that they have no conflict of interest.

Ethics approval: All samples were recovered in accordance with local ethical approvals (R.S.44.20), from “Tor Vergata” University COVID-Hospital of Rome.

Consent to participate: Informed consent was obtained from all subjects enrolled in the study.

Consent for publication: Informed consent was obtained from all subjects enrolled in the study.

Availability of data and material: Not applicable

Code availability: Not applicable

Authors' contributions: M. Nuccetelli coordinated the study and all the experiments; M. Nuccetelli and A. Cristiano wrote the manuscript; M. Nuccetelli, A. Cristiano and V. Fortunati analyzed data, performed the experiments and revised the final version; F. Cherubini and S. Bernardini critically revised the paper. All the authors approved the final version of the paper.

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Table

	Negative controls (N=44)	Positive early infection patients (N=44)	Positive late infection patients (N=48)
IgG anti-prothrombin	5.110 U/ml (range 2.720-11.20)	5.475 U/ml (range 3.960-11.20)	5.020 U/ml (range 0.920-45.20)
IgM anti-prothrombin	1.405 U/ml (range 0.007-7.630)	1.150 U/ml (range 0.100-3.660)	1.220 U/ml (range 0.007-20.00)
IgG anti-annexin-V	4.967 U/ml (range 3.023-10.80)	4.933 U/ml (range 2.507-8.467)	3.638 U/ml (range 0.900-23.75)
IgM anti-annexin-V	2.812 U/ml (range 1.190-5.433)	2.957 U/ml (range 1.537-5.067)	1.531 U/ml (range 0.075-16.35)
IgG CICs	1.045 RU/ml (range 0.1000-10.30)	0.66 RU/ml (range 0.1000-3.940)	1.120 RU/ml (range 0.2900-9.660)

Table 1 IgG/IgM anti-prothrombin, IgG/IgM anti-annexin-V, IgG CICs median concentration and range

Figures

Figure 1

IgG anti-prothrombin results in control group, positive early infection group and positive late infection group are shown in panel A (KRUSKAL-WALLIS p value=0.1938); IgM anti-prothrombin results in control group, positive early infection group and positive late infection group are shown in panel B (KRUSKAL-WALLIS p value=0.3584)

Figure 2

IgG anti-annexin-V results in control group, positive early infection group and positive late infection group are shown in panel A (KRUSKAL-WALLIS p value=0.0101); IgM annexin-V results in control group, positive early infection group and positive late infection group are shown in panel B (KRUSKAL-WALLIS p value=0.0029)

Figure 3

IgG circulating immune complexes (CICs) results in control group, positive early infection group and positive late infection group (KRUSKAL-WALLIS p value=0.0008)