A comparative study between CLIA serological assay and Real-Time PCR analysis to detect SARS-CoV-2 in Albanian Clinical Practice

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Abstract

Text SARS – CoV-2 is a pathogenic coronavirus which continuously evolve as changes in the genetic code occur during replication of the genome. Real-Time PCR analysis is the main standard for identification of SARS-CoV-2- infection. Because there are limits in its utilization for large-scale screening, serological assays have been used for detecting SARS-CoV-2 presence.

The aim of this study is to compare and highlight the efficiency of these methods in COVID-19 diagnostics. Nasopharyngeal samples were collected from 1198 patients which were analyzed after with RT-PCR. 90 of these patients resulted positive with SARS-CoV-2 virus and 55 of them were subjected to CLIA serological assay. As a result, 49 (89.1%) patients were positive only for IgG, 4 (7.3%) patients were positive for both antibodies IgG and IgM and only 2(3.6%) patients were negative for both antibodies, based on serological results. However, both assays had better performance 8-10 days after symptoms appearance, meanwhile the serological assay was more predictable at least 10 days after symptoms appearance. The serological assay used in this study helps in a better monitoring of the patients whom resulted positive with RT-PCR, especially to define the infection stage. As a conclusion, serological assay is limited in usefulness when diagnosing SARS-CoV-2 infection but it is useful to provide information in patient's immunoreaction to COVID-19 exposure. A combination of both molecular analysis and serological assays is the most effective way to diagnose this virus.

Keywords: SARS-CoV-2; Covid-19; CLIA serological assay; RT-PCR

1. Introduction

Coronaviruses are large, enveloped viruses with linear positive-strand RNA genomes 1 that cause mainly respiratory tract infections in humans. Previously, there have been two pandemics caused by SARS-CoV (WHO - World Health Organization 2020; CDC (Center for Disease Control and Prevention 2021)) and Middle East respiratory syndrome coronavirus (Mizumoto *et al*., 2019; Hartley *et al*., 2020) and both demonstrated a high risk to global public health. Since December 2019 globally and since March 2020 in Albania, there has been an outbreak of the new coronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 causes severe respiratory tract infection and is transmitted rapidly from contacts with people (Price *et al*., 2021; Dinnes *et al*., 2020). Thus it is important to be diagnosed quickly in order to prevent transmission and break the

contamination chain. There are several methods to diagnose SARS-Co-V-2 which are often intertwined with each other. Molecular techniques detect the genetic material of SARS-CoV-2 and this detection principle is based on the nucleotide bases pairing specificity in homologue strands (Yang& Rothman, 2004; Dinnes *et al*., 2020). Real-time Reverse Transcription PCR method is the golden standard for diagnosing SARS-CoV-2 because it is a very sensitive method (Goudouris 2021; Shen *et al*., 2020). But still it has its limitations, as it is difficult to carry out swab tests for the whole population in Albania, with the number of certified and authorized laboratories for research of viral RNA and the relatively long time required to generate results. Thus, there is still a demand in finding novel and rapid assays to diagnose COVID-19.

Serological assays for COVID-19 diagnostics detect IgG, IgM and IgA antibodies in serum. They detect a present or past infection by monitoring the disease progress (Jacofsky *et al*., 2020). CLIA (Chemiluminescent Immunoassays) detects the antigen-antibody immunologic complexes where the analytic reaction is tagged by a luminescent molecule (Mohit *et al*., 2021). The serum IgA, IgM and IgG are captured in magnetic particles covered with SARS-CoV-2 antigens (N or RBD). The chemiluminescent immune-analysis is an automated assay and is used to analyze a large number of samples. Serological analysis steps are less complicated and do not request previous (Machado *et al*., 2021).

Both analysis, molecular and serological assays are not efficient if they are performed during the first week of infection because the virus is still in incubation period and as a result there are not enough viral RNA copies detectable via RT-PCR nor are yet created SARS-CoV-2 antibodies (Jarvis & Kelley, 2020; Rode *et al*., 2021). The symptoms begin almost 5 days after the contact with the virus and this phase corresponds to the window period. The best time to perform real time RT-PCR is approximately one week after the beginning of symptoms or two weeks after the contact with the virus, as in this period the viral load is really high (Loeffelholz & Tang, 2020; Falzone *et al*., 2021). The best time to perform a serological assay for both antibodies is approximately 4 weeks after the contact with the virus (Falzone *et al*., 2021).

Antigen tests, biochemical analysis and imagery techniques can be also used to diagnose and monitor the patient. Antigen analysis discover the presence of a specific viral antigen, like S protein and N protein (Nguyen *et al*., 2020). A various number of laboratory parameters can evaluate the acuteness of the disease, like C protein (CRP), procalcitonin (PCT) and D-dimer (Letelier *et al*., 2021; Eastin & Eastin 2020). Among radiologic techniques, chest X-Rays (CRX) and computerized tomography (CT) are the most efficient to diagnose pneumonia caused by COVID-19 (Li & Xia 2020).

The aim of this study is to compare molecular and serological assays used to diagnose SARS-CoV-2 and to evaluate the importance of each method in our country.

2. Material and Methods

The study was conducted at Genius laboratory in Tirana, during March-June 2021. 1198 nasopharyngeal samples were analyzed with RT-PCR to identify the positive cases with SARS-CoV-2. Nasopharyngeal swabs were obtained from the nasopharyngeal cavity of the patients.

The *alphaPrep*^{TM} kit (Viral DNA/RNA Extraction Kit Model: VRD-B096V)) was used to extract RNA from nasopharyngeal samples, which contained lyophilized buffer proteinase K and a PCR plate with 96 wells filled previously with buffer and magnetic beads. The PCR plates were placed in the Automated Nucleic Acid Extraction System NC-15 PLUS HanwoolTPC Co., Ltd, which consists in automatically extracting RNA.

2.1. *Molecular analysis*

For real time RT-PCR analysis, GeneFinder^{TM} Covid-19 PlusReal*Amp* Kit was used which detects the three genes of SARS-COV2-, RdRp, E and N. This kit contains a mixture of Tris HCl, MgCl2, dNTPs,

reverse transcriptase enzyme and Taq DNA polymerase, and a mixture of probes and the negative and positive controls.

Table 1. Amplification conditions of real time RT-PCR

	Phases	Temperature	Time	Cycles
1	Reverse transcription	50 ℃	20 min	1
2	Pre-denaturation	95℃	5 min	1
3	Denaturation	95ºC	15 sec	45
	Bonding	58ºC	60 sec	

Table 2. Fluorescent dye for target genes

	Phases	Temperature	Time	Cycles
1	Reverse transcription	50 ℃	20 min	1
2	Pre-denaturation	95℃	5 min	1
3	Denaturation	95°C	15 sec	45

15 µl of the master mix prepared, RNA sample extracted from each patient and the negative and positive control were added to each well of the PCR plate. The stripes were centrifuged in a micro-centrifuge and then were placed in the Applied Biosystems® QuantStudio™ 7 Flex Real-Time PCR System thermocycler.
Real Time RT-PCR analysis was performed according to the cycling program of QuantStudio-Real Time PCR software v1.3. Cycling conditions are shown in table 1 and the fluorescent dye for target genes are shown in table 2. Samples were considered positive at molecular screening if all three genes were detected.

2.2. *Serological assay*

CLIA immunoassay was used as a serological technique to analyze patient's blood serum. Blood samples were collected in CLIA Activator standard microtubes. They were centrifuged for ten minutes at 4000 rpm and then the serum supernatant was collected. The kit used to detect IgM was MAGLUMI TM 2019-nCOV IgM (CLIA) Snibe Diagnostics. This kit has magnetic nanoparticles coated with antihuman monoclonal IgM antibodies and recombinant 2019-nCOV antibodies tagged with ABEI, an activated ester which is widely used as a chemiluminescent tag in monoclonal antibody mixtures for immunological tests. MAGLUMI SARS-CoV-2 S-RBD IgG (CLIA) Snibe Diagnostics kit was used to detect IgM, with nanoparticles conjugated with the antigen SARS-CoV-2 S-RBD and with IgG antihuman antibodies.
The serologic test was performed in the automated MAGLUM 4000 plus SNIBE Diagnostics analyzer (Shenzhen New Industries Biomedical Engineering Co., Ltd, Shenzen, China). Starter 1 & 2 were also used, in order to generate the chemiluminescent signal for detection. The light signal is measured by RLU, which is proportional with the concentration of the sample antibodies. According to the instructions of the kit, for antibody values lower than or equal to 1Au/ml the results are positive. If IgM and IgG value is lower than 1Au/ml the result is considered negative or not detectable.
The data processing and the descriptive statistics were analyzed with SPSS program. Chi-square, Mann Whitney and Kruskal Wallis statistical tests were performed.

3. Results and Discussions

3.1. *RT-PCR results*

An amplification graphic with Ct values of the genes is obtained for each patient that had a real time RT-PCR test done. Out of the 1198 samples analyzed with real time RT-PCR, 90 (7.5%) of them were positive for SARS-CoV-2 and 1108 (92.5%) patients were negative.

Table 3. Distribution of positive cases based on gender

Real Time RT-PCR result		
Gender	Positive frequency (%)	Negative frequency (%)
Female	47(52.2%)	637 (57.5%)
Male	43(47.8%)	471 (42.5%)
Total	90(100%)	1108 (100%)

Out of 1198 patients that underwent the real time RT-PCR test, 684 were females and 514 were males. From 90 individuals that resulted positive, 47 (52.2%) of them were females and 43 (47.8%) were male. It resulted that there is no significant change in the number of affected females and males (Chi-square $X^2(1)=0.943$, $p=0.331$).

Real time RT-PCR analysis is capable to identify infection only in the active phase of SARS-CoV-2, meanwhile serologic tests are capable of showing an infection which has already passed (Krajewski *et al*., 2020). The viral load can be under the detection threshold, which is why this analysis can produce false negative results (La Marca *et al*., 2020). The best time to obtain samples for analyzing is approximately one week after symptoms start or two weeks after first contact with the virus because at this period the viral load is very high in the upper respiratory tract (Loeffelholz &Tang, 2020; Falzone *et al*., 2021). During this period false negative results are minimal. After the viral charge has reached its peak, it starts to reduce and symptoms begin to relieve as well. Still, at this period the probability for a false negative result increases (Jarvis & Kelley, 2020).

3.2**.** *Serological test results*

Out of 90 patients that resulted positive from RT-PCR, 55 of them did the serologic test after two weeks. It resulted that 49(89.1%) patients were positive only for IgG, 4(7.3%) were positive for both antibodies and only 2(3.6%) were negative for IgM and IgG.

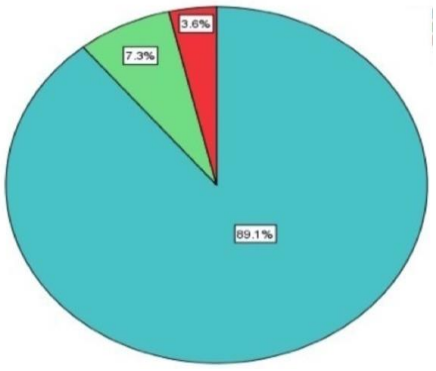

Figure 1. Presence of patient's antibodies

Thus, four patients resulted positive for IgM, meanwhile there were not detected these antibodies for the other 51 patients. Detection of both antibodies in this case shows that these positive patients for SARS-CoV-2 are still in the active phase of infection. Jacofsky *et al*.,2020, noticed that when infection continues the body produces both antibodies actively. The combination of molecular and serologic assays shows that routine diagnosis for COVID-19 is very important. Molecular analysis confirms the virus presence and the serologic test plays an important role in prognosis and disease progression (Wu *et al*., 2020). Also, concerning IgG, 53 patients were positive and two patients resulted negative for this antibody. Most of the patients who were confirmed positive from real time RT-PCR, resulted positive only for IgG after two weeks and this result shows that they are in the late stages of infection or in the recovery period.

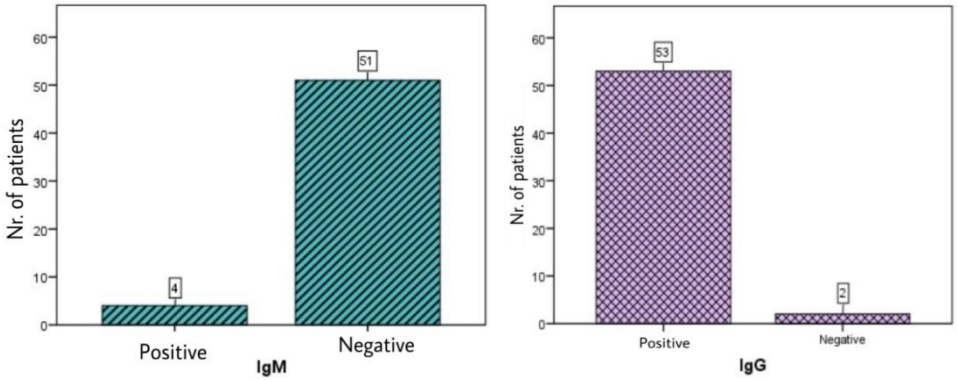

Figure 2. IgM and IgG positivity levels

Only two patients, in this study, had neither antibodies in their system. One of the reasons might be because some people produce antibodies three weeks after infection or even later. Another reason is immunosuppression or age factor (CDC, 2020; Rode *et al*, 2021). The two patients were both females aged 77 & 87. The immune system is weak at an old age and antibodies require more time to differentiate (Bajaj *et al*., 2020). This shows that serologic tests cannot be used as a primary assay without previously verifying them using RT-PCR because it is not a method that detects the presence of the virus right away. According to our study, one out of 16 infected people had no IgG antibodies and this is a disadvantage to serologic tests (Petersen *et al*., 2020). The serologic assay is more valuable as a complementary test to RT-PCR, which is why it is important in diagnostics (Zhao *et al*., 2020). Quantitative analysis for IgM and IgG play an important role in diagnosis, evaluation and prognosis of COVID-19 (Hou *et al*., 2020). According to Milani *et al*., 2020 individuals with aggravated symptoms of the virus, develop a stronger immune response and both antibodies are at high concentration in these patients. Ghaffari *et al.*, 2021 states that knowing the dynamic of the immune response is crucial to formulate diagnosing and treatment strategies. IgM studies are important because it is the first class of immunoglobulins that are produced when our organism is exposed to a pathogen. IgM stability is much lower than that of IgG in the serum (Qu *et al*., 2020). In comparison to IgM, IgG is an antibody which is very specific in neutralizing pathogens, that is why its study is very important. Usually, IgG is produced in later stages of an infection and it plays an important role in creating long-term immune memory, even though it is not known yet the stability of these antibodies after the infection with SARS-CoV-2 (Nguyen *et al*., 2020). Gluck et al., 2020 noticed in his study that IgG were detectable in 90% of individuals, 30 weeks after the appearance of symptoms and IgG levels remain relatively stable for at least six months. Quantitative detection of IgG has a great impact in research in order to understand if the created antibodies protect us from future infections and how long

will this immunity last (Dan *et al.*, 2021).

3.3. *Serological analysis according to age and gender*

The results of our study showed that out of 55 patients, 28 of them were female and 27 were male. Four females tested positive for IgM levels and IgG was detected in only one male and female patient. According to Hossain *et al*., 2021 and Lai *et al*., 2020, seroprevalence of IgG and IgM in females and males is the same, thus indicating that gender is not a decisive factor in disease progression (Hossain *et al*., 2021; Lai *et al*., 2020).

Average IgG levels in females resulted 43.1643 Au/ml with a standard deviation of 32.78776 Au/ml and in males it was 47.0259 Au/ml with a standard deviation 43.660952 Au/ml. Even though in figure 3 it seems that males have a higher level of antibodies, it results that there was no significant change on the antibody levels between the two genders (Mann Whitney U=364.500, p=0.820). This result is similar to another study (Luo *et al.*, 2021). Males might have higher levels of antibodies because infected males with SARS-CoV-2 have increased inflammatory responses that might cause the body to produce more B cells, therefore they produce more antibodies (Korte *et al*., 2020).

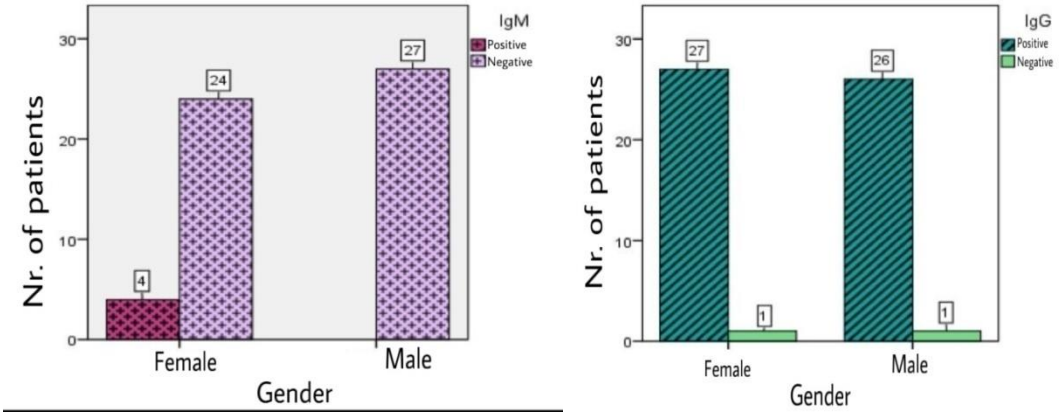

Figure 3. IgM and IgG seropositivity based on gender

The average age of 55 individuals that were submitted to the serologic test was 43 ± 16.997 years old, meanwhile the average age of individuals that were submitted to the test was 31-35 years old. Age frequencies are shown in the graph below (figure. 4). IgM antibodies were detected in three patients that were 20-30 years old and in one 79 year old patient. All of the patients between the age range 20-30, 31-45, 46-59 were positive for IgG. Only two patients of the age 77 & 87 tested negative for this antibody.

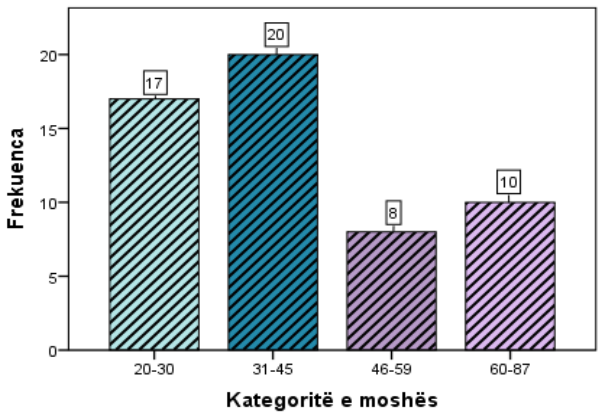

Figure 4. Age groups frequencies

IgM levels had no significant changes in different age groups (Kruskall Wallis $X(3)^2=2.348$, p=0.503). IgG levels had significant changes between age groups where the lowest level was detected in the group ages 60-87 years old (Kruskall Wallis $X^2(3)=9.586$, p=0.022). However it should be taken in consideration that there were fewer patients in the age range 46-59 and 60-87 years old.

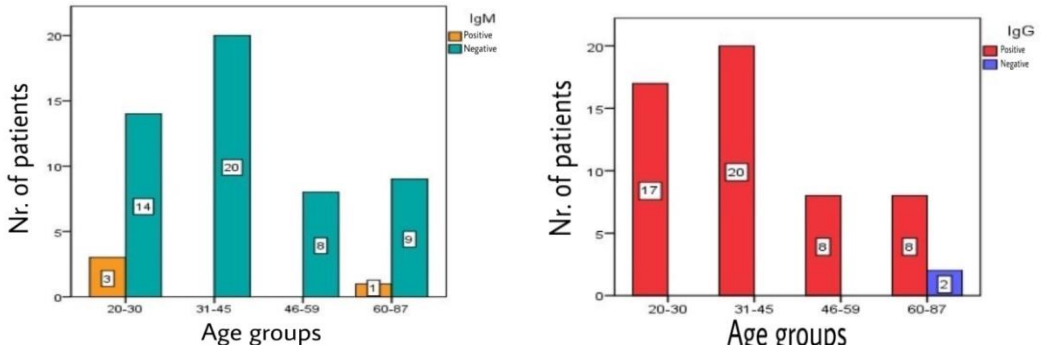

Figure 5. IgG and IgM seropositivity based on age

According to Luo *et al*., 2021, in a study where the dynamic level change of IgG and IgM were evaluated, it resulted that during the first 30 days, antibody levels were lower in older group ages and higher in younger group ages. The high level of antibodies in young ages during the first stages of the infection might play an important role in preventing the disease from aggravating. However in other studies, it is noticed that older ages produce higher antibody levels in later stages of infection compared to younger ages (Klein *et al*., 2020; Luo *et al*., 2021). This was also noticed in our study where most of the patients who were confirmed positive with real time RT-PCR, after two weeks had created IgG in their serum and this actually shows a late stage of infection or the stage of healing. Serologic tests were also used to identify potential donors for plasm therapy and to evaluate the immune response in candidate vaccines (Winter & Hegde,2020). Combining molecular analysis with serologic tests is very important in routine diagnosis of COVID-19. Molecular analysis confirms the virus presence and the serologic test plays an important part in evaluating the progression and

prognosis of the infection (Wu *et al*., 2020). Two patients, in our study, had not created any antibodies for SARS-CoV-2. This indicates that serologic test cannot be used alone without real time RT-PCR confirmation because it is not a diagnostic method that detects the presence of the virus. Serologic test is an indirect method that detects the presence of antibodies that have been created. Furthermore, Peterson et al., 2020 noticed in their study, that in 1 out of 16 people infected, IgG antibodies were not present and this is a disadvantage for serologic tests. Real time RT-PCR is the golden standard to diagnosing SARS-CoV-2 because it is very specific and it detects the genetic material of the virus directly in its active phase of infection (Goudouris 2021). The serologic test is very valuable as a complementary analysis and a great help for real time RT-PCR, which is why routine application of the serologic test in diagnosis and clinical management is important (Zhao *et al*., 2020). In this study all of the patients that did the serologic test resulted positive for SARS-Cov-2. The serologic tests are very important for epidemiologic studies because it limits the spreading of SARS-CoV-2, evaluating the real prevalence of the infection in the entire population. Real time RT-PCR gives false negative results for patients that are in the convalescence period and for asymptomatic patients that are characterized by a low viral charge (Borges *et al*., 2020).

4. Conclusions

RT-PCR method is effective to diagnose early stage SARS-CoV-2 in its active phase of infection and it cannot be replaced by serologic tests. Meanwhile CLIA is an indirect method that detects IgG and IgM. Serologic tests cannot be used to confirm SARS-CoV-2. Thus, application of serologic methods is clinically important in helping RT-PCR. These tests help monitor patients that have resulted positive from RT-PCR, to see if patients are healed or if they are in the active phase of infection. Based on the result of this study, females and males are equally affected by SARS-CoV-2 and ages 60-87 have the highest rate of infection. Levels of IgM and IgG has no significant change from females to males and IgG levels are lower at ages 60-87 years old.

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Tables

Table 1. Cyclation conditions of real time RT-PCR

Table 1 shows amplification conditions and cycles of real time RT-PCR

Table 2. Fluorescent dye for target genes

Table 2 shows the temperature, time and cycles of fluorescent dye for target genes

Table 3. Distribution of positive cases based on gender

Table 3 shows that out of the 1198 samples analyzed with real time RT-PCR, 90 (7.5%) of them were positive for SARS-CoV-2 and 1108 (92.5%) patients were negative. Out of 1198 patients that underwent the real time RT-PCR test, 684 were females and 514 were males. From 90 individuals that resulted positive, 47 (52.2%) of them were females and 43 (47.8%) were male.

Figures

Figure 1. Presence of patient's antibodies

Figure 1 expresses graphically the presence of patient's antibodies. Out of 90 patients that resulted positive from RT-PCR, 55 of them did the serologic test after two weeks. It resulted that 49(89.1%) patients were positive only for IgG, 4(7.3%) were positive for both antibodies and only 2(3.6%) were negative for IgM and IgG.

Figure 2. IgM and IgG positivity levels

Figure 2 shows respectively the positivity levels of IgM and IgG. Thus, four patients resulted positive for IgM, meanwhile there were not detected these antibodies for the other 51 patients. Also, based on IgG levels, 53 patients were positive and two patients resulted negative for this antibody. Only two patients, in this study, had neither antibodies in their system.

Figure 3. IgM and IgG seropositivity based on gender

Figure 3 expresses graphically the seropositivity of IgG and IgM based gender. The results of our study showed that out of 55 patients, 28 of them were female and 27 were male. Four females tested positive for IgM levels and IgG was detected in only one male and female patient.

Figure 4. Age groups frequencies

Age group frequencies are shown in figure 4. IgM antibodies were detected in three patients that were 20-30 years old and in one 79 year old patient. All of the patients between the age range 20-30, 31-45, 46-59 were positive for IgG.

Figure 5. IgG and IgM seropositivity based on age

IgG and IgM seropositivity for each based on patients age is shown in figure 5. IgM levels had no significant changes in different age groups. IgG levels had significant changes between age groups where the lowest level was detected in the group ages 60-87 years old.