The Investigation of P1 gene in Mycoplasma pneumonia Isolated from atypic pneumonia by molecular methods, determine IgG antibody and MIC to ciprofloxacin antibiotic

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Abstract

Objective

Mycoplasma pneumoniae, which causes atypical pneumonia, is a well-established pathogen of the respiratory tract. This bacteria is intrinsically susceptible to fluoroquinolones. But Recently, drug-resistant forms of this bacteria have been reported. This study aims to determine the prevalence of this bacteria by ELISA and PCR and MIC to ciprofloxacin.

Methods

The clinical samples (blood and nasopharyngeal swab) were collected from 100 patients who were referred to selective hospitals in Tehran with respiratory complaints were enrolled in 2017.Nasopharyngeal swab sample collections were cultured on PPLO broth and PPLO agar. After culturing and DNA extraction, PCR was performed by specific P1 genes primers. Ciprofloxacin's MIC of *Mycoplasma pneumonia* isolated was determined by Micro-broth dilution method. Also, measure serum IgG antibody titers were measured by ELISA Mycoplasma pneumonia.

Results

In this study, out of 100 samples 12, bacteria were isolated on PPLO agar.Using specific primers,7 samples of Mycoplasma speciesism-specific were positive for the presence of *M.pneumoniae*. 2 Ciprofloxacin resistant isolates were evaluated. ELISA results show that the IgG titre antibody In 19 samples is existent. Also,5 samples are intermediate. IgG antibody titre average in the whole sample is 27/66 U/ml but it is in Positive samples by P1 PCR is 45/75 U/ml.

Conclusions

This study showed that PCR is a sensitive and reliable method for rapid detection of *M. pneumoniae* bacteria in respiratory infectious samples.but the results of this method are different from the ELISA method. also, It seems that the Resistance to ciprofloxacin is relatively common among *M. pneumoniae*.

Introduction

M. pneumoniae is one of the main etiological agents causing atypical pneumonia, especially in children and young adult [1–3].It accounts for as many as 10–30% of all cases of community-acquired pneumonia (CAP)[2, 4] .Epidemiological studies in China have demonstrated that *M. pneumoniae* infections account for 20.7% in adult cases, more than Streptococcus pneumoniae, and turn out to be the leading pathogen of CAP[2]. Although most of these infections are mild and often self-limiting, severe bronchopneumonia and lung abscesses can occur. Furthermore, *M. pneumoniae* infections may also lead

to several extra-pulmonary conditions, such as myocarditis, pericarditis, meningitis, neuritis, and hemolytic anemia, sometimes with fatal outcomes [5–7].

Because treatment of *M. pneumoniae* infections with β-lactam antibiotics is ineffective and the clinical manifestations of *M. pneumoniae* infections are complicated and nonspecific, a rapid, sensitive, and specific laboratory test is vital for early diagnosis of *M. pneumoniae* infection [8, 9] .Conventional tests, including cultivation and serological methods, have their limitations in detecting *M. pneumoniae*. For example, *M. pneumoniae* culture is difficult, time consuming, and lacks sensitivity, and is therefore not recommended for clinical practice[4, 10] .Serological methods are currently the most common tool used in the clinical laboratory. However, these methods have practical limitations because of the availability of paired serum samples from both acute and convalescent phases, and provide results of questionable specificity and sensitivity[10–12] .

Both the *P1* adhesin gene and *16S rRNA* gene have been utilized widely in PCR techniques as the targets for detection of M. pneumonia [13–15]. The *P1* adhesin gene is an intriguing target gene for PCR because of its repetitive nature within the genome[14]. About 8% of the *M. pneumoniae* genome consists of repetitive DNA elements with regions homologous to the *P1* adhesin gene, thus allowing an increase in the sensitivity of a PCR assay [16].

Several bacterial surface proteins, including 170-kDa protein, *P1*, are involved in the formation of the attachment organelle and cytadherence of *M. pneumoniae* to the respiratory epithelium. The 170-kDa protein *P1* is a majora adhesin protein which is densely clustered at the site of the attachment organelle[17] .Protein *P1* is encoded by a gene of nearly 5,000 bp, comprising copies of repetitive regions RepMP2/3 and RepMP4, which are present in the *M. pneumoniae* genome in 10 and 8 copies, respectively[18] .

The *16S rRNA* gene is also an attractive candidate as a target due to its unique organization and the presence of conserved and variable regions on its abundant high-copy number[14, 19].As for which of them is the better choice, there is still no uniform standard.

Since mycoplasmas lack a cell wall, the number of antibiotics that can be used for treating mycoplasmal infections is limited. Antibiotics with potential activity against mycoplasmas and used in clinical practice include the tetracyclines, MLSK antibiotics and fluoroquinolones[20, 21]. These drugs achieve high intracellular concentrations in mammalian cells and are thereby able to reach the intracellular mycoplasmas. Only the fluoroquinolones and ketolides have a potential bactericidal action. All three classes have the advantage of being active against other bacteria that may be associated with mycoplasmas in respiratory and genital tract infection.

The first members of the fluoroquinolone class, such as ofloxacin and ciprofloxacin were followed by new agents, like levofloxacin and moxifloxacin. Newer fluoroquinolones show an enhanced activity against all the human mycoplasmas studied including *M. pneumoniae*, compared with the older ones. However MICs of all fluoroquinolones are higher than those of macrolides and related antibiotics [22].

The aim of this study was to measure the presence of this bacteria by molecular and ELISA methods, evaluate the presence of *P1* genes in isolates, also, IgG antibody titer against this bacteria was also measured by ELISA method and Finally, the minimum inhibitory concentration(MIC) is determined by the antibiotic ciprofloxacin (as common fluoroquinolones.

Materials And Methods

Strain collection and sampling

In this study, 100 people with pneumonia were hospitalized and samples were taken in selected hospitals in Tehran. The disease was confirmed by a physician based on criteria such as clinical symptoms, chest radiography, and patient history. Sampling was performed in June-February 2017.

Using a sterile disposable syringe (Yazd syringe), blood samples were taken from all individuals and poured into the test tube containing clot activator. The sterile swab (Dacron® swab) in the test tube containing the transfer medium was used to sample the pharynx. The transmission medium used is PPLO broth (BBL medium - China). Throat swabs were prepared from patients. The swabs were transferred to the laboratory for culture after numbering in the vicinity of ice (4 degrees Celsius(.

Selection of specific mycoplasma pneumoniae culture medium and culture of isolated samples

PPLO agar and PPLO Broth (BBL medium - China) were used to cultivate the samples. The selection of these two culture media was based on the availability of manufacturing facilities and the availability of a growing medium. To prevent the growth of bacteria in the sample, penicillin, amphotericin B and polymyxin solutions should be added to PPLO Broth culture medium. The solution for these solutions should be prepared before the start of the production process, also, D-Glucose solution must also be added to the culture medium for bacterial growth.

Isolated isolates must be cultured after the *M. pneumoniae* culture medium has been cultured and the necessary ingredients have been added. Cultivation of *M. pneumoniae* For this purpose, in sterile conditions, using a syringe, one milliliter of the culture medium was removed and transferred to PPLO Broth solution by 0.45 micron filter of the syringe head with low pressure. The culture medium was monitored at 37 degrees Celsius and incubator for 21 days. During this time, color changes (color of the reagent) or turbidity were monitored. After 21 days, 1 ml of each medium was added to the secondary culture medium (the third regrowth from the time of sampling). PPLO agar plate was added from all 0.5 ml culture media. The plates were transferred to a 37 degrees Celsius incubator. The incubator contained a CO2 atmosphere (5-10% concentration). The plates were evaluated daily for 3-7 days. Bacterial growth status was evaluated using 10X optical microscopy with magnification was re-cultured from the transition medium.

PCR molecular test

Bacterial preparation:

PPLO Broth was poured 0.5 ml of culture medium (re-culture medium) into the 1.5 ml microtype. This was done using a sterile sampler. The microtubules were centrifuged at 13,000 rpm for 15 minutes (Sigma-Germany). The supernatant was removed and bacterial sediment was used to extract the DNA. In order to positively control the DNA extracted from the bacterium *M. pneumoniae* (29342: ATCC-produced by Razi Institute) and for negative control, a culture medium without bacteria was used.

DNA Extraction

Genomic DNA for subsequent PCR was done using the Roche kit and taking into account the kit instructions.

Preparation primer

The sequence of primers for the *P1, 16SrRNA* genes specific for the *M. pneumoniae* species used in previous studies was evaluated. This assessment was performed using Oligo Primer Analysis Software version 7. After the approval of the desired primers, the order to make the primers was sent to Sina Cloon. The sequence of primers used in this study is presented in Table 4. It should be noted that the PCR reaction is performed to confirm the molecular identity of the isolates using the P1 Mycoplasma pneumoniae-specific primer, while the reaction was performed to determine the bacterial abundance in the swab using a *16SrRNA*-specific Mycoplasma primer.

PCR process

Molecular detection of *P1, 16SrRNA* and was carried out according to the following condition:

initial denaturation at 72 °C for 5 min followed by 35 cycles of denaturation at 72 °C for 45 s, annealing at 63 °C for 40 s, and extension at 95 °C for 45 s, and final extension step at 95 °C for 5 min.

The sequences of all used primers are shown in (all reactions performed in duplicate and along with the negative control (water) and positive (previously known positive-PCR products) control. The positive DNA control bacterium *M. pneumoniae*, produced by the Razi Institute, was also used for its qualitative control. The final products were detected by electrophoresis on 1% agarose gel containing DNA safe stain (Sinacolon, Iran). The marker used in the SMO BIO survey was made by Cena Cloon with a distance of 100 pairs of open weights from 100 to 3000 pairs.

MIC test

After preparing a special culture medium for this test, the bacteria are prepared.

Dilution microbial method requires a bacterial solution with a certain concentration. If the concentration of the bacterium is not known, the amount of inhibition of bacterial growth will not be accurate. In order to determine the MIC inside the test tube, a very high concentration (comparing the turbidity with the McFarland scale) is used. However, such a concentration is not possible for the bacterium *M. pneumonia*.

For this purpose, a suspension containing 10^4-10^5 CFU is used. This suspension is prepared using the colon count method on PPLO Agar environment. Bacteria that were positive for *P1* genes were cultured and the number of CFUs in the reference suspension was calculated using a reverse microscope. The CFU in bacterial suspension was then adjusted accordingly.

After the preparation of the bacteria, the antibiotic solution ciprofloxacin was prepared. According to the CSLI 2010 table, the antibiotic potency of ciprofloxacin was 50%. The amount of antibiotic powder required to prepare 1 ml of ciprofloxacin solution was calculated according to the CSLI table.

For this purpose, 2048 micrograms of ciprofloxacin powder was dissolved in 1 ml of 98% ethanol (it is difficult to measure weight on a microgram scale, so a concentrated solution was prepared and then volume was added). Dilution of the solution was performed using pure water.

A 96-well plate well was used for bacterial culture for MIC test.

In order to investigate each sample (bacterial culture), 19 wells were considered. For this purpose, the first well was considered as a positive control containing 150 µl of PPLO broth medium and 150 µl of bacterial suspension. (Growth in this well indicates that the bacterium is active.)

The second well is diluted at 270 microliters from the PPLO broth culture medium and 30 microliters from the antibiotic suspension. Subsequent wells up to the 18th well contain 150 microliters of PPLO broth. From the second well, 150 µl of solution was transferred to the third well after complete pipetting. The transfer of the solution from the previous well to well 18 continued. 150 microliters will be discarded from the 18 well solution. 150 µl of the bacterial suspension was added to wells 2–18 and each half 0.5 µl of bacterial suspension. 300 µl of PPLO broth culture medium was added to well 19. Well 19 plays the role of Blanc.

Determination of specific IgG antibody titer of Mycoplasma pneumonia:

For this purpose, the serum was first separated from the patients' blood using centrifugation and kept at -20 Celsius until further steps.

In this study, Kate-EUROIMMUN (made in Germany) was used to perform the ELISA test.

The test was performed using a quantitative method (due to greater accuracy). This method is used to measure the antibody level of *M. pneumoniae*. The test was performed using the instructions in the kit, and finally the standard kit curve was used to calculate the curve results (reading results). Accordingly, values less than 16 units per milliliter were considered as a negative result, and between 16 and 22 as an intermediate result. Values greater than 22 units per milliliter were also considered positive results. The results were read using Eliza Reader (Biotech-USA).

Statistical analysis

In this study, information was evaluated using SPSS software (IBM-USA-version 12). For this purpose, Spearman, Anova, Chi-square and T-tests (to compare the results of the two methods) were used. A significant criterion for statistical differences was P values smaller than 0.05.

Results

Culture result

Among the cultured samples, 12 bacterial isolates with egg and strawberry morphology were obtained. The structure of the half-egg on the PPLO culture medium is shown in Fig 1.

PCR results for the 16srRNA and the P1 gene

In order to determine the contamination of the specimens, bacteria of the genus Mycoplasma PCR were performed. DNA was extracted from all samples. A special *16srRNA* primer made of mycoplasma was used. The piece is about 713bp long (based on the primer structure). Accordingly, 14 samples infected with Mycoplasma bacteria were considered. The PCR image of a piece of the *16srRNA* gene of the Mycoplasma pneumonia is shown in Fig 2.

PCR of the *P1* gene was performed to confirm the molecular identity of the clones obtained. Due to the specificity of the primer at the cheek surface, PCR results testify to the presence of mycoplasma pneumonia. After DNA extraction and proliferation of the *P1* gene, it was proved that all isolated bacteria are *M. pneumonia*. Based on the results obtained from PCR, the identity of *M. pneumonia* was confirmed in 7 isolates. Fig 3 shows an example of the results of 450bp proliferation of the P1 gene of *M. pneumonia*.

Results of the population indicators of people infected with M. pneumonia

In this study, various population indicators of people infected with *M. pneumonia* were studied. These indicators included smoking, coughing, sinusitis and asthma, and muscle aches.

1. In connection with the smoking, Using a square statistical test, the overall difference between tobacco use and non-smoking in infection caused by pneumococcal mycoplasma was determined. p-value= 0.671 obtained indicates that there is no significant statistical difference between the consumer group of tobacco and the group without consumption.
2. Based on the type of cough, the participants in this study were divided into four subgroups (dry cough, whooping cough, no cough, whooping cough and whooping cough). The statistical relationship between cough and infection with *M. pneumoniae* is presented in Table 1. Therefore, in this study, the occurrence of dry cough, which is one of the symptoms of *M. pneumoniae*, has no statistical relationship of infection with this bacterium.
3. Among people infected with *M. pneumoniae*, 2 (28%) people with sinusitis were evaluated and among those without infection, 10 (10.7%) people with sinusitis were evaluated. In terms of

statistical test, these values do not differ (p-value = 0.62).

4. Among the participants in this study, 5 people with asthma were evaluated. None of the people infected with *M. pneumoniae* had asthma.
5. Muscle pain is one of the extrapulmonary symptoms of *M. pneumonia*. However, this symptom is not specific to the *M. pneumoniae*, and other pneumonia-producing organisms indicate this symptom. In this study, out of 100 patients evaluated, 27 reported muscle pain complaints, of which 4 were infected with pneumococcal mycoplasma and 23 were non-infectious. Kai's square statistical test showed that there was no association between muscle pain and infection with mycoplasma pneumoniae (p-value = 0.06). However, this result is significantly closer.

Mic result

11 *M. pneumoniae* isolates were evaluated using microdermabrasion method for microbial resistance to ciprofloxacin. Of the 7 mycoplasma pneumoniae isolates, 2 (28%) isolates were evaluated as resistant to ciprofloxacin. Table 2 and Fig 4 presents the results of this test.

ELISA result

Based on the results of the ELISA test, the specific IgG antibody titer of *M. pneumoniae* was evaluated positively among 19 of the 100 participants in this study. There were also 5 intermediaries. The lowest serum level among these individuals was 1 unit and the highest titration rate was 98. Fig 5 shows the frequency distribution of IgG antibodies in the subjects in this study.

Fig 6 also shows the frequency distribution of different IgG antibodies to pneumococcal antimicrobial IgG.

.Comparison of antibody titers and PCR

7 cases were evaluated for culture and PCR infection infected with *M. pneumoniae*. Antibody titer in these individuals is presented in Table 3. The table also includes the headlines of people who were positive for Mycoplasma infection.

Discussion

M.pneumoniae is a bacterial pathogen without a cell wall of the respiratory tract that infects the respiratory tract. The bacterium is also one of the leading causes of acquired pneumonia in the community. The most common manifestations of *m.pneumonia* include flu-like symptoms such as sore throat, fever, cough, headache, chills, colds, muscle aches, and general discomfort. *M.pneumoniae* infection occurs worldwide and is most common in children and adolescents. *M.pneumoniae* is transmitted primarily from close contact with another person. In recent years, pneumonia caused by mycoplasma has increased. Pneumonia caused by *M.pneumoniae* occurs through damage to the airways of the lungs in a contagious manner and inflammation caused by *M.pneumoniae*.

The pneumonia caused by this bacteria is generally resistant to treatment with beta-lactam antibiotics. It depends on the structure of the bacterium because it is unable to produce peptidoglycan. However, allergies to other antimicrobial agents are seen in this bacterium. However, resistance to antibiotics in this bacterium has been discussed in many societies. If left untreated, some patients may develop the disease [23].

Data from more than 21 countries showed that *m.pneumoniae* is the most common type of bacterium that causes atypical pneumonia, which is responsible for creating about 12% of the acquired pneumonia in the community. Past studies on *m.pneumoniae* have shown that they are 40% more likely to develop acquired pneumonia.. A recent cross-sectional study of people with atopic pneumonia showed generally lower data rates than in any other country. Climate differences, seasons, and improved environmental and public health are likely to reduce the risk of contracting the bacterium. In general, because it is difficult to diagnose mycoplasma biochemistry, little research is available on other bacteria. The slow growth and susceptibility of the bacterium can also double the accuracy and sensitivity of the techniques used to diagnose it.

The main strategy of this study, according to its title, was first to answer the question of what is the prevalence of *m.pneumoniae* in people with atopic pneumonia. The next step was to measure the drug's resistance to ciprofloxacin in the resulting isolates. The first goal (frequency determination) was performed in three different ways. Cultivation method, PCR molecular method and ELISA method.

In the present study, we joined orthogonal experiment and single-factor tests to optimize several crucial factors in PCR assay based on both the *16S rRNA* gene and *P1* adhesin gene designed for *M. pneumoniae* detection.

Gene targets used widely in various types of PCR assays for *M. pneumoniae* include the *P1* adhesin gene and *16S rRNA* gene[13]. The *P1* adhesin gene is an attractive target for PCR because it repeats up to 10 times within the *M. pneumoniae* genome, which increases the sensitivity of PCR assay[16]. Another important target is *16S rRNA*, or rather rDNA. The advantage of using rDNA sequences is the high degree of conservation of the target and the presence of the highly variable regions[14] .Therefore, it would be greatly valuable for guiding treatment decisions and follow-ups, particularly in countries with a high frequency of strains resistant to antibiotics, because the confirmation of *16S rDNA* could be applied in discriminating between bacterial and viral causes of pneumonia.

To our knowledge, however, it is still controversial which target is more effective. For instance, Loens et al. suggested that the *P1* adhesin gene may be more sensitive than the *16S rRNA* one[13]. Two independent researchers, nevertheless, showed that the amplification of the *16S rRNA* gene was more sensitive for the detection of *M. pneumoniae* because more positive samples were found by *16S rDNA* PCR than by a PCR with the *P1* gene[14, 15]. The main reason for the ambivalent conclusions is that the researchers detected the DNA directly from clinical samples, rather than a standard strain DNA of *M. pneumonia*, to compare the sensitivity of *16S rDNA* PCR with *P1* gene PCR.

These results confirmed that the *16S rRNA* gene primers are more sensitive than the *P1* adhesin gene primers, as the *16S rRNA* gene primers can detect up to 10 fg of *M. pneumoniae* DNA and the *P1* gene primers can detect 100 fg of *M. pneumoniae* DNA at most. This was mainly because the presence of approximately 103 copies of *16S rRNA* per mycoplasma cell and the high degree of conservation of the rRNA genes allowed a high fixation of primers on the target and lead to a higher PCR yield[4, 14] .Importantly, because RNA is destroyed more rapidly than DNA after the death of the mycoplasma cell, detection of the *16S rRNA* gene provides further evidence of viable mycoplasmas in the specimen [4].

At this stage, part of the *16SrRNA* gene of this bacteria was replicated. Propagation was performed using mycoplasma-specific primers. The second step was to use the PCR technique using specific *P1* gene primers.

Primer F has a general structure, but primer R (return) has a specific structure for screening for mycoplasma. In 2014, Tabatabai Qomi et al. Used these primers to examine the presence of mycoplasma as a contaminant in cellular categories. In addition to *M.pneumoniae*, they identified other bacteria of this genus. Bacteria including Mycoplasma arginini, Mycoplasma hyorinis, Mycoplasma orale, Mycoplasma synoviae, Mycoplasma gallinarum were also identified[24] .

We observed that 0.14% (14/100) of the specimens were positive with the *16S rRNA* gene PCR and 7% (7/100) of the specimens were positive with the *P1* adhesin gene PCR.

The first use of these primers dates back to 1995 and research by Pruckler et al. The aim of this study was to study the species of *M.pneumoniae* that can be traced in cellular categories [25].

In 2014, Mirihan et al. Used this primer to study the infection with Mycoplasma bacteria in patients with pneumonia admitted to Egyptian hospitals. They assessed a prevalence rate of 22.94% among 170 individuals. In the present study, a prevalence of 14% was observed, which is lower than that of Mirihan et al. The reason for this decrease is probably related to Iran's health conditions [26].

Determination of *M.pneumonea* species was also determined by specific proliferation of *P1* gene. The results of this study showed that 7 samples are infected with *M.pneumoniae*. Another 7 samples were positively evaluated for mycoplasma. The identity of the infected species remained unknown.

In 2015, Amirian et al. In Saveh city used PCR method to diagnose *M.pneumoniae* using *P1* gene proliferation. Out of 120 people with chronic lung infection, 8 were positive, with the highest frequency in the 21-year-old age group and the lowest in the 3-year-old age group [27].

The positive PCR result is based on the *16SrRNA* gene, but the lack of bacterial growth is due to the origin of the microbial agent. The bacteria is present and is made of mycoplasma but is unable to grow. Yoshida et al., Using similar primers and replicating part of *16SrRNA*, isolated the specific species of birds related to the genus Mycoplasma [28].

Cultivation is rarely used for diagnosis of *M. pneumoniae* infection in most clinical laboratories because the fastidious growth requirements and length of time necessary to culture *M. pneumoniae* (three to six weeks) make growing the organism impractical for patient management[4, 10].

Currently, serological assay is the most widely used means for laboratory confirmation of mycoplasmal respiratory infections [10]. However, there are concerns about the use of single qualitative tests to identify acute *M. pneumoniae* infections in adults, since many persons may not mount an IgM response, presumably because of re-infection, and when it is produced, IgM may persist for long periods [19, 29, 30]. Furthermore, the percentage of individuals with acute infection who demonstrated a positive IgG response in the acute phase was less than 50% in a recent study.

It has been suggested that cross-reactivity with antigen preparations used in some of the commercial enzyme immunoassays (EIAs) result in over-diagnosis of *M.pneumoniae* infections[29]. Therefore, even if serology is a useful epidemiologic tool in areas where the infection rate of *M. pneumoniae* is high, it is less suited for assessment of individual patients in clinical laboratories[11]. Compared with serology and culture, a direct detection of pathogens in clinical specimens has been done more regularly using molecular biology techniques. PCR approaches have been the most valuable method for rapid, sensitive, and specific diagnosis of *M. pneumoniae* infection[4]. However, the application of molecular methods of enhanced sensitivity may be necessary since the pathogens are probably present in small quantities.

In 2003, Orsi et al. Evaluated the performance of four PCR-based *M.pneumoniae* identification methods. In this study, false PCR results were confirmed. This study proves that bacterial culture is the most reliable way to measure mycoplasma in terms of false positives. However, based on this study, the false negative results of the cultivation technique are high[13].

In 2006, Shah Hosseini et al., Using culture and PCR methods, compared the performance of these two methods to identify pneumococcal mycoplasma in people with acquired pneumonia. According to their research, all cases of positive culture in terms of PCR will also be positive. Therefore, PCR was considered an accurate and hypersensitive method of culture [31].

In 2013, in a study using serology and PCR, Noorbakhsh etal.Studied the role of *m.pneumoniae* in children with adenoid hypertrophy and rhinosinusitis. Positive PCR results were reported in 35% of the samples. A survey of antibody titers by ELISA also found that 10 percent of people with IgM and 20 percent had IgG in their serum. In this mycoplasma, pneumonia was also detected using PCR in adenoid tissue samples[32] .

In the present study, data from the ELISA method showed that out of a population of 100 people studied, 19 contained specific anticoagulants of *M.pneumoniae*. This rate is negligible compared to many other studies. In 2002, for example, Rastawick et al. Found that 63% of the 66 patients admitted to Polish hospitals had specific IgG antibodies specific to *m.pneumoniae* [23]. This high level is probably dependent on factors such as the antibody titer determination kit.

In addition, reviewing the ELISA results and comparing it to the PCR test reveals a valuable point. All seven samples infected with *M.pneumoniae* were also positive for ELISA. This shows the importance of positive ELISA results. However, species of the genus Mycoplasma (other than Mycoplasma pneumoniae) were unable to increase its antibody titer.

in 2001, Suni et al. Used the ELISA method to investigate the prevalence of Mycoplasma pneumoniae infection in people with acute and non-acute pneumonia. They showed that 92 out of 210 people tested positive for IgG mycoplasma pneumoniae in their serum [33].

Sharifı et al. In 2011 in Tabriz, using PCR, culture and ELISA methods, examined the frequency of respiratory infections caused by *M.pneumoniae*. In addition, they measured patients' IgM antibody titers. Out of 200 patients with pneumonia, 12 samples were evaluated by PCR method infected with this bacterium. Also, 4 samples were isolated by *m.pneumoniae* bacterial culture method. Using ELISA technique, it has been proven that there are 10 mycoplasmal IgM antibodies in the serum of individuals [34].

In 2004, in Tehran and in Tehran, Niakan et al. Used the ELISA method to investigate the serological frequency of IgG associated with *m.pneumoniae* in people with atypical pneumonia. ELISA method was used in their research. Blood serum was assessed in 104 individuals, of which 57% were positive. 36% were negative and 5% were intermediate [35].

In this study, samples tested positive for *M.pneumoniae* were evaluated for MIC in terms of ciprofloxacin. The result of this evaluation was resistance 2 separation. Restriction of *m.pneumoniae* in treatment (intrinsic resistance to beta-lactam antibiotics) has led to the treatment of this bacterium as a sensitive issue. There is an increase. For example, 80 to 90 percent prevalence of macrolide resistance leads to ineffectiveness as one of the most important and effective factors in their treatment [36].

On the other hand, research on *m.pneumoniae* and its antimicrobial properties is much more difficult than other bacteria. Therefore, the main focus is on the limited scientific reports provided from various sources. This has led to the fact that these reports, like the report from the present study, are of particular importance. The prevalence of 2 resistant isolates among the 8 resistant isolates (in the present study) compared to the prevalence reported in the Gruson study, and colleagues reported the resistance of this bacterium against resistant strains. It had the highest resistance among all antibiotics. The maximum reported MIC for ciprofloxacin in the Gruson study was 128 micrograms per milliliter. And the minimum MIC value was 1 microgram per milliliter. The fact that the bacterium Mycoplasma pneumoniae is more resistant to ciprofloxacin than other antibiotics is a warning sign of an increase in its prevalence. However, in our country, this increase in resistance is not seen. To confirm this, we can refer to the research of Kashmiri et al. In 2014. In general, the prevalence of resistance is 2 isolates less than 7 isolates than the 81% resistance of macrolides in Japan [37]and less than 7.5% of the resistance of levofloxacin in Europe (France) [38].

In 2011, Aizhen et al. Investigated the therapeutic response to concomitant use of ciprofloxacin and corticosteroids in the treatment of infections caused by *m.pneumoniae*. They found combination therapy to be effective. However, some people experience delayed fever and symptoms that appear to be genetically predisposed. This study shows that the patient's genetics are involved in the symptoms and treatment of pneumococcal mycoplasma [39].

In 2014, Biljana et al. Examined the role of community-acquired pneumonia created by M.pneumoniae. 166 children aged 1 to 15 years were assessed. In this study, serological methods and RT PCR technique were used. Specific IgM and IgG antibodies to *m.pneumoniae* were measured in patients' serum. During this study, the clinical symptoms of *M.pneumoniae*, including headache, wheezing, were evaluated. It was also found that 14.5% of people with *m.pneumonia* [40].

Conclusion

M.pneumoniae is one of the smallest release and pathogenic organisms. This bacterium has problems in diagnosis and treatment. The inability to perform biochemical tests on it doubles the need for epidemiological studies. The next important thing about this bacterium is its treatment. There have been recent reports of widespread resistance to *M.pneumoniae*. Antibiotic-based treatment has led to widespread concern about the spread of this resistance among the scientific community. In the present study, the diagnosis of pneumococcal mycoplasma was made using *P1* gene culture and PCR method. Although the results of this experiment were lower than the ELISA results, the ELISA did not have a false positive. In this study, 14 isolates based on PCR gene of *16SrRNA* gene were identified as a part of mycoplasma species. PCR was then used to identify the *P1* gene, which resulted in the identification of seven species. Thus, 7 other species were evaluated as non-Mycoplasma pneumoniae species. A survey of the questionnaire showed that 9 out of 14 people are related to birds. The presence of domestic birds and contamination with the *M.pneumoniae* are also statistically related. Ciprofloxacin MIC testing showed that the 2-isolate bacterium is resistant, which is not very significant compared to other antibiotic reports.

Declarations

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Author contributions

ZD, MN, MK: design of study. ZS, MK, HA, SJ: acquisition of data. ZD, MN, ZD, SJ: evaluation of data, preparation of the manuscript. ZD, MN, MK, HA : assessment of data. All authors read and approved the final manuscript.

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Availability of data and materials

All relevant data are included in the manuscript.

Ethics approval and consent to participate

This study was in accordance with the declaration of Helsinki. This study was approved by the Ethics Committee of Shahed University of Medical Sciences. (Ethical code: IR.SHAHED.REC.1397.039). The informed consent was obtained from all the participants, and informed consent obtained was written.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Tables

Table 1 Frequency distribution of cough and infection with M.pneumonia.

Dry cough and sputum	Cough with sputum	Dry cough	No cough	
0	3	4	0	Infected with bacteria
17	41	26	9	Not infected
0/214	0/950	0/104	0/388	P-value

Table 2 MIC values of pneumococcal mycoplasma isolates

Antibiotic	MIC (micrograms per milliliter)	Sample Code
Ciprofloxacin	015/0	MD11
	003/0	MD22
	007/0	S4
	015/0	CH19
	007/0	M93
	506/0	MD75
	012/1	MD62

Table 3 Compare antibody titers and PCR

Sample code	Pollution status	Antibody-unit header	Gender
MD11	Confirmation of Mycoplasma pneumonia	43/6	M
MD22	Confirmation of Mycoplasma pneumonia	41/9	F
S4	Confirmation of Mycoplasma pneumonia	64/8	M
CH19	Confirmation of Mycoplasma pneumonia	36/2	M
M93	Confirmation of Mycoplasma pneumonia	57/1	M
MD75	Confirmation of Mycoplasma pneumonia	39/9	F
MD62	Confirmation of Mycoplasma pneumonia	28/7	F
MD68	Confirmation of mycoplasma	40/6	F
MD34	Confirmation of mycoplasma	25/4	M
M43	Confirmation of mycoplasma	32/3	F
B51	Confirmation of mycoplasma	25/4	F
E12	Confirmation of mycoplasma	9/1	M
R4	Confirmation of mycoplasma	13/9	F
B41	Confirmation of mycoplasma	14/66	M

Table 4 Sequence of primers used in this study

Gene	Product size	Primer sequence	Reference
16S rRNA	713 bp	F: 5- ACTCCTACGGGAGGCAGCAGT - 3' R: 5- TGCACCATCTGTCACTCTGTTAACCTC - 3'	[41]
P1	450 bp	F: 5- AAAGGAAGCTGACTCCGACA - 3' R: 5- TGGCCTTGCGCTACTAAGTT -3'	[42]

Figures

Figure 1

Half-egg structure on PPLO culture medium.

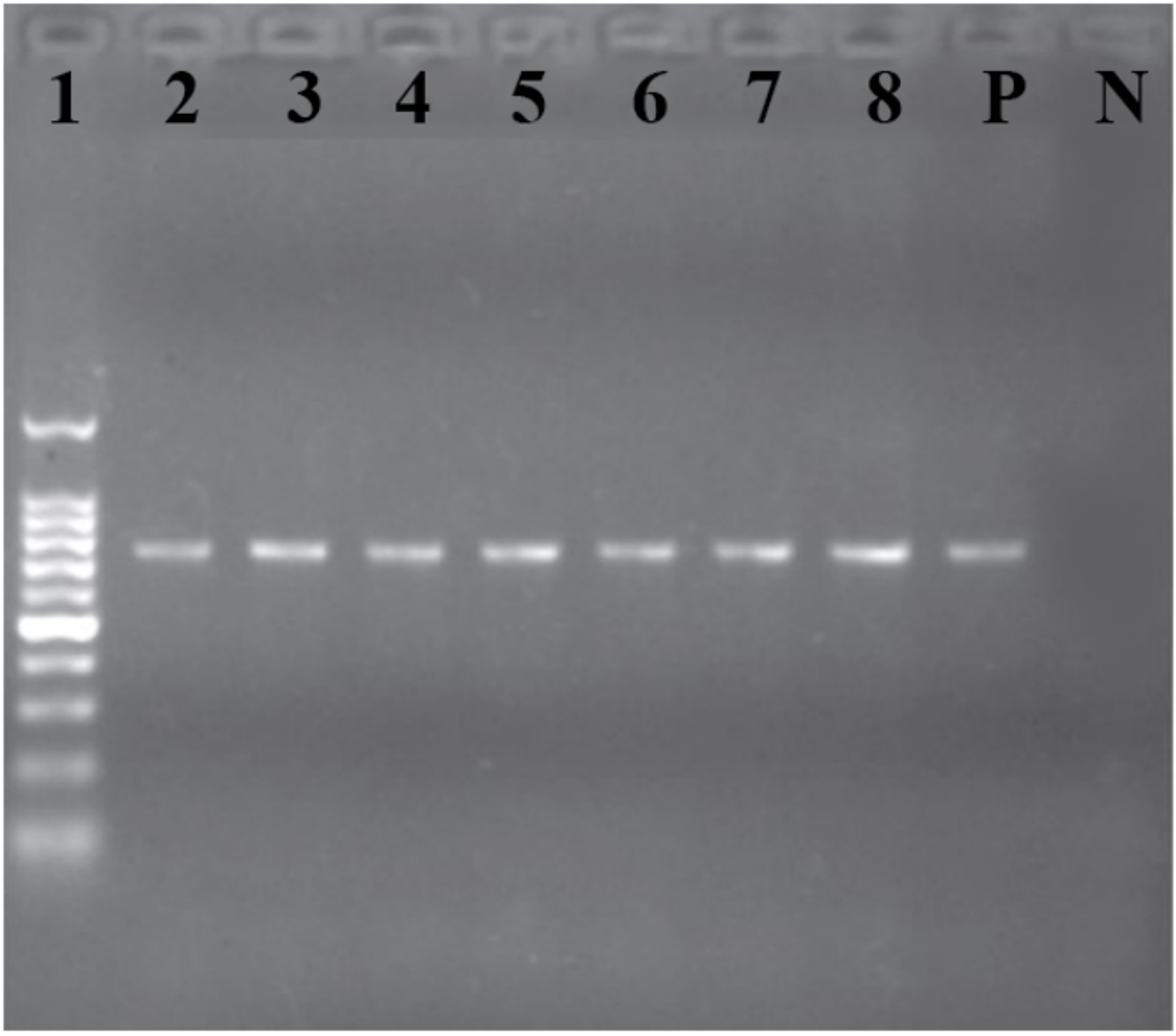

Figure 2

PCR product piece of the 16srRNA gene of the M.pneumoniae. P: Positive Control. N:NegativeControl. 1:Led Marker

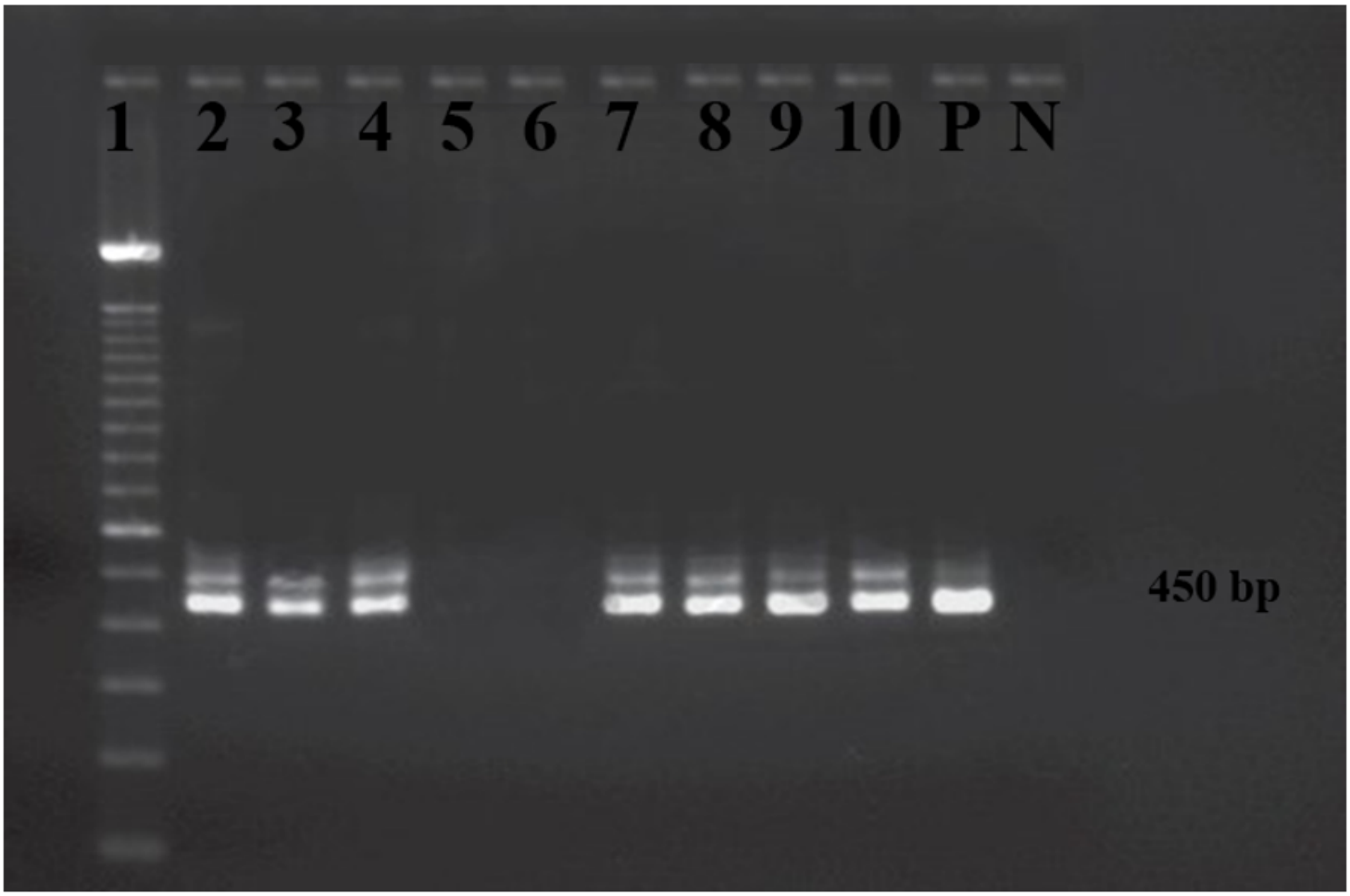

Figure 3

PCR product electrophoresis gel fragment of the P1 gene of M.pneumoniae. P: Positive Control. N: Negative Control. 1: Led Marker.

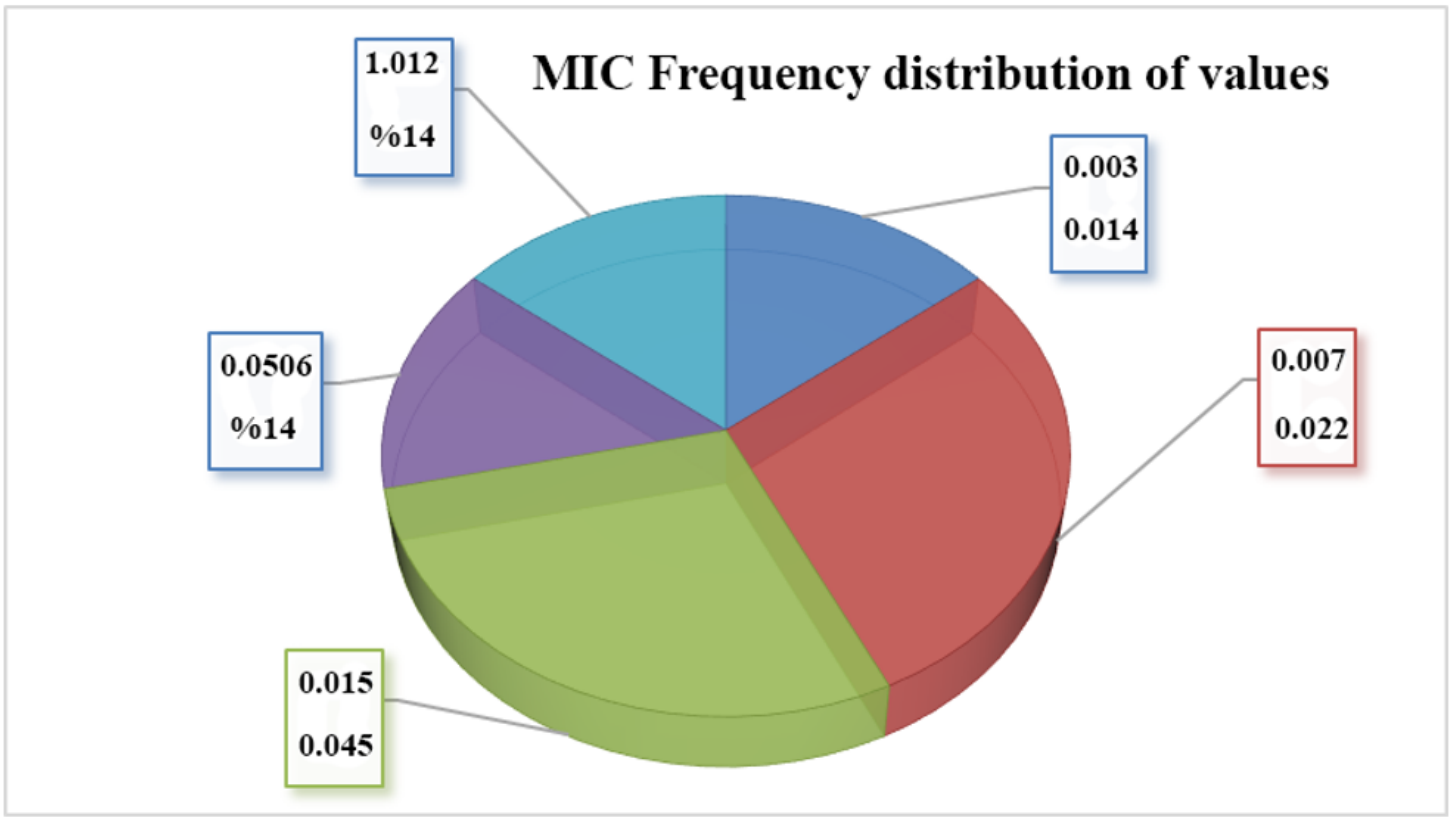

Figure 4

Abundant distribution of MIC in this study (values in micrograms per milliliter)

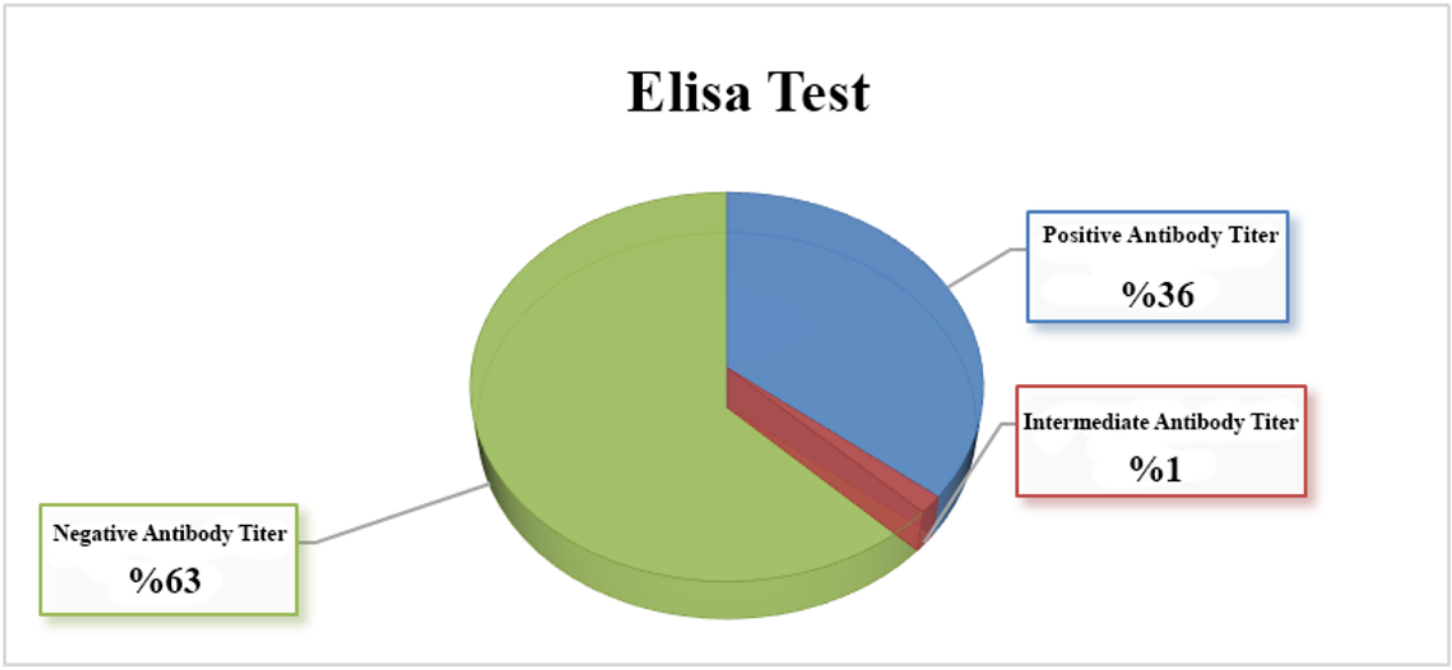

Figure 5

Specific IgG antibody to M.pneumoniae

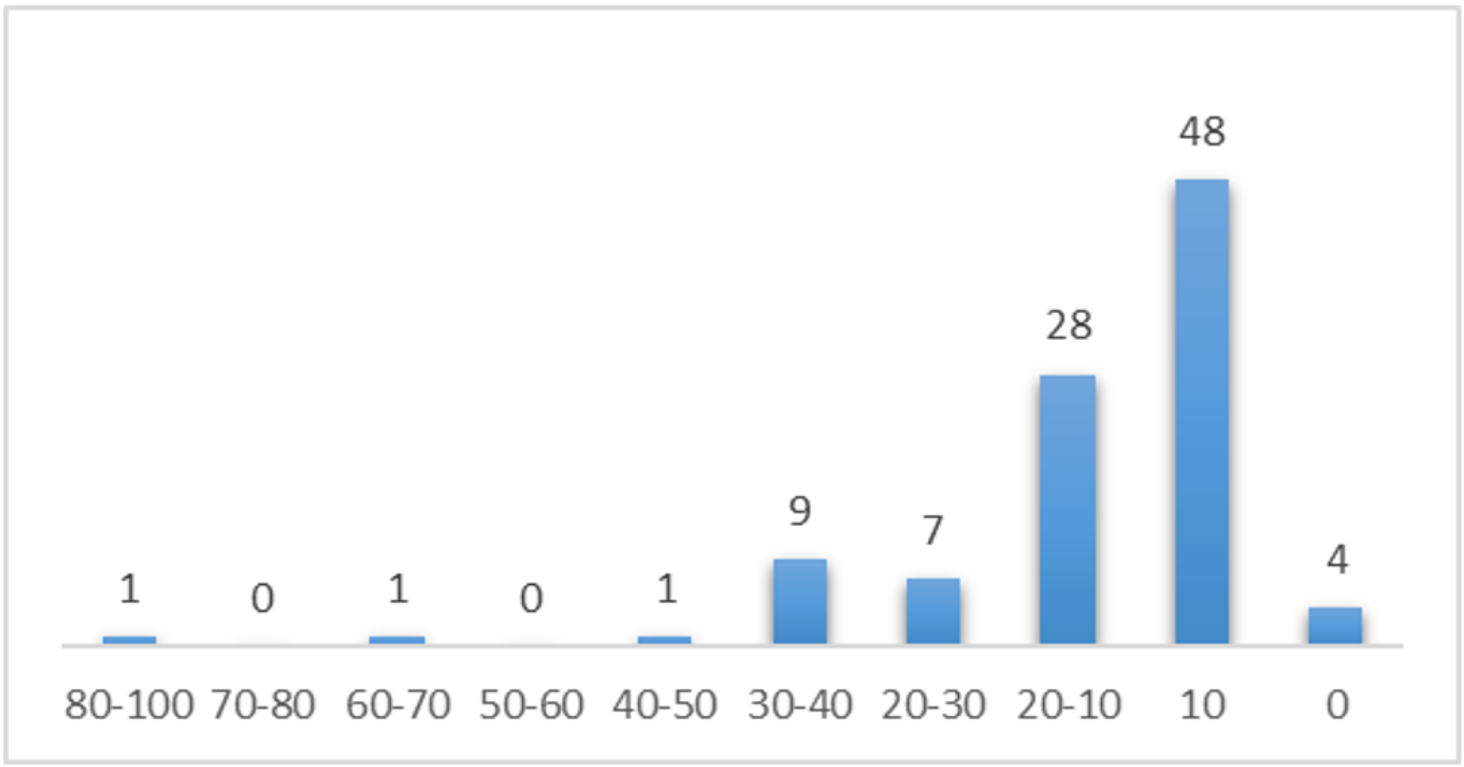

Figure 6

Frequency distribution of various IgG antibodies against M.pneumonia