J. Appl. Environ. Biol. Sci., 4(10)243-249, 2014

ISSN: 2090-4274
Journal of Applied Environmental and Biological Sciences
www.textroad.com

Isolation and Identification of Mycoplasma Pneumonia's Molecular Nature from Patients with Chronic Pulmonary Infections

Maryam Sheikh Zadeh[1,*], BabakKheirkhah[2], Ashraf Karimi Nik[3], Ferdows Sheikh Zadeh[4]

[1]M.Sc. Department of Microbiology, Kerman Science and Research Branch, Islamic Azad University, Kerman, Iran
[2]Assistant Professor, Department of Microbiology, Kerman Science and Research Branch, Islamic Azad University, Kerman, Iran
[3]Assistant Professor, Department of Microbiology, Kerman Branch, Islamic Azad University, Kerman, Iran
[4]MPH. Department of Public Health, Kerman University of Medical Science, Kerman, Iran

Received: June 8, 2014
Accepted: August 11, 2014

ABSTRACT

Background and goal: the role of Mycoplasma Pneumonia in chronic pulmonary infections has been identified. These infections cause inflammation responses and chronic pulmonary infections. This study has been conducted on patients with chronic pulmonary infections who have been sent by the specialist to laboratory for tuberculosis diagnosis and their polymerase chain reaction (PCR) for mycobacterium was negative.
Materials and methods: in this descriptive study, sputum specimens of the patients with chronic pulmonary infections was taken and sent to the laboratory for authentication of mycobacterium species. After PCR test, those mycobacterium negative, were asserted for Mycoplasma Pneumonia's genus and species identification by means of specific primers.
Results: 36 percent of 50 mycoplasma-negative specimens were positive and showed 139 base pair band. 66 percent out of these positive samples were infected by Mycoplasma Pneumonia and showed 465 base pair specific band.
Conclusion: the results of this study has indicated that one quarter of patients with chronic pulmonary infections who, by words of the specialist, are prone to tuberculosis, are not infected with mycobacterium and mycoplasma's genus has been isolated from the sputum transduction.
KEYWORDS: Mycoplasma Pneumonia; chronic pulmonary disease; tuberculosis; molecular nature identification

INTRODUCTION

One of the diseases that chronically involve lungs is tuberculosis. Tuberculosis is one of deadly diseases, which is caused by mycobacterium or specifically, tuberculosis mycobacterium. This contagious disease is of hygienic problems in underdeveloped countries.(1) lungs are the victims in tuberculosis, which is called T.B. Tuberculosis, but other organs of the body i.e. central neurone system, lymph nodes and blood circulatory system, genial and urinal system, gastrointestinal system , bones , joints and skin can be involved in this disease, too. (2) Researches in Iran show that only 10 percent of the patients which tuberculosis symptoms who are referred to laboratory by specialists, are mycobacterium positive and therefore another factor has caused chronic and severe symptoms, which, mycoplasma is of utter importance among bacterial causes.(3). This microorganism is of common causes for upper and lower respiratory system infections and is considered as normal flora in respiratory and genital-urinal ducts but their role in causing infections in these areas has been identified. (4, 5) *Mycoplasma Pneumoniae* is of important and common bacteria for acute respiratory infections and peripheral pneumonia. Researches show that it is more common in autumn and winter. The symptoms include headache, enervation, fever (38.9 – 39.4 °C), and sore throat, dry and sudden cough which is followed by sputum and extra pulmonary manifestations are uncommon. (6).This organism, along with Chlamydia, is of asymptomatic Pneumonia causing factors which, its respiratory infections often leads to singular or family epidemic disease. (7). It seems that one of the main causes of uncommon pneumonic syndromes is this bacterium which scatters or causes epidemics. Half of the causes are because of *Mycoplasma Pneumoniae* and the microorganisms are involved in the other half. (8).nonetheless, because of the culturing and isolation problems of these bacteria in laboratories and the organisms 'role in human infection and the upcoming side-effects, they have been neglected in our country. (9), but similar related studies have been conducted. Especially studies related to isolation and using polymerase chain reactions (PCR) for identification of *Mycoplasma Pneumoniae* in patients with respiratory system infections symptoms. (7,10). Molecular techniques such as PCR has the potential to identify this pathogenic factor accurately and fast and therefore, can provide the antibiotic treatment

[*]**Corresponding Author:** Maryam Sheikh Zadeh, M.Sc. Department of Microbiology, Kerman Science and Research Branch, Islamic Azad University, Kerman, Iran

for the patient sooner. (10,11). The aim of this study is isolation and identification of the molecular nature of *Mycoplasma Pneumoniae* from non-pneumonic patients stricken with chronic pulmonary infections in Kerman in the first 6 month of the year in 2013.

MATERIALS AND METHODS

In this descriptive study, objective samples were taken from all of the patients with chronic pulmonary infections who have been sent by the specialist to authenticated molecular center for tuberculosis diagnosis in Kerman in the first 6 month of the year in 2013. By considering all of the hygienic and medical-related criteria and by means of professional staff, sputum samples were taken from these patients and actions were taken for genus and species of the agent bacteria and its molecular nature identification. In this study, samples were not enriched and cultured, as PCR is so complete and accurate a method for isolation the agent. 0.5 ml of the sample was put in shaker and then was transferred to 1.5-ml micro tube and then was centrifuged (13000 rpm) for 15 minutes. Then the supernatant was evacuated and gene extraction was done on 100µl pellet.

A) Gene extraction:

Cinna-clone Company's extraction kit under the name of

CAT NO: PR881613

Cinna Pure-DNA (Cell culture, Tissues, Gram negative Bacteria and CSF), 50 Preps

Was used according to its manual for gene extraction . Then, until the PCR stage, the extracted DNA was put in freezer.

B) Polymerase chain reaction (PCR) :

In this study, first, fragments of oxyR gene were used as target gene for detecting the sputum-existing mycobacterium. Primer sequence and its temperature characteristics are shown in Table 1. The positive and negative controls used in PCR were standard DT mycobacterium strain and sterile distilled water, respectively.

Table 1: primers used for amplification the 547base pair fragment from oxyR gene of the mycobacterium in suspected samples

Target gene	Primer	Oligonucleotide sequence (5′–3′)	Fragment size (bp)	Annealing temperature
oxyR	F	5′-GGTGATATATCACACCATA-3′	548	55
	R	5′-CTATGCGATCAGGCGTACTTG-3′		

After verifying that the samples are mycobacterium-negative, fragments of 16S rRNA of mycoplasma's gene was used as targeted DNAs separately for mycoplasma genus and *Mycoplasma Pneumoniae* species detection. The positive and negative controls used in PCR were standard DT mycobacterium strain and PPLO broth medium, respectively. Nucleotide sequences an characteristics of the primers used for mycoplasma genus and *Mycoplasma Pneumoniae* species identification in this study have been shown in Table 2, 3.

Table 2: Nucleotide sequences and characteristics of the primers used for mycoplasma genus by means of PCR

Target gene	Primer	Oligonucleotide sequence (5′–3′)	Fragment size (bp)	Annealing temperature
16S rRNA	F	5′-GGCGAATGGGTGAGTAACACG-3′	465	61.59
	R	5′-CGGATAACGCTTGCGACCTATG-3′		

Table 3: Nucleotide sequences and characteristics of the primers used for *Mycoplasma Pneumoniae* species by means of PCR

Target gene	Primer	Oligonucleotide sequence (5′–3′)	Fragment size (bp)	Annealing temperature
16S rRNA	F	5′-GGCGAATGGGTGAGTAACACG-3′	465	61.59
	R	5′-CGGATAACGCTTGCGACCTATG-3′		

The synthesized primers were lyophilized from the factory and were bought from Cinna Gene Company and each primer pair was treated as the instructions given in the accompanied leaflets. First, each vial of the primer was mixed with 500 µl distilled- and –without- DNAase enzyme -water which was bought from the same company and after some vortexes, it was mixed and dissolved. The next step was providing stock from this mixture. For that, specific amount of the main primer mixture (which was solved in 500 µl), was diluted with sterile distilled water to 50µl volume.

For making the Master Mix or the main mixture, 10x ‘ dNTPs ‘ $MgCl_2$ buffer, primer and enzyme were mixed near ice in a 0.5 ml tube and after that, sample’s DNAs were added separately to the master mix. PPLO Broth medium and *Mycoplasma Pneumoniae* standard sample DNA (NCTC 10123) were used as negative and positive controls, respectively. Also, in one of the wells, marker, which is used for measuring the weight of the bond, was poured. The other wells respectively included: marker in first well, positive control in second well, negative control in third well and wells number 1-8 were for the samples. The important point is that the master mix was shaken many times before it is distributed into tubes. Also, the 25µl-micro tubes were shaken before moving to the thermal cycler. The specific program related to PCR (according to the genus and species of the detected bacteria) was given to the thermal cycler to be used.

C) Examination of PCR production:

Gel electrophoresis of the PCR productions was used for asserting the productions of PCR qualitative and semi-quantitatively, presence or absence and/or the amount of products. The gel which was used in this study was 1% and regarding to this, the agarose powder was measured and TBE buffer was added. (1g agarose powder in 100 ml TBE solution) first, the appropriate container and ladder was chosen and then, they were washed with distilled water and dried. After that, 2 sides of the container were sealed with tape in order to prevent the liquid agarose out before its solidification. Then, the container was put in a balanced and still place and the ladder was adjusted in it. An Erlenmeyer flask containing gel and TBE buffer was put in microwave oven, so that the powder dissolves and a transparent liquid is achieved. After cooling, the gel was dyed with Ethidium bromide (0.5 µg dye for 1 ml gel). The liquid gel was poured into the container and after its solidification, the tape and ladder were removed, therefore, wells are constructed in gel. After that, gel was put in the electrophoresis tank containing buffer and samples were dyed with Loading Dye Buffer (2 part dye: 10 part sample) on aluminum foil paper and then, 3.5 to 3.7 µl of the samples, controls and 100pb marker were load in wells. (It is advised to load the marker in first well).at the end of the electrophoresis stage, gel was removed from buffer, washed in water, put in UV-transilluminator and images and prints were taken out of it.

RESULTS

First, by conducting PCR test on sputum specimen, 50 suspicious and mycobacterium-negative samples were chosen. The image of positive samples which were not included in the studied population is shown in Figure 1.

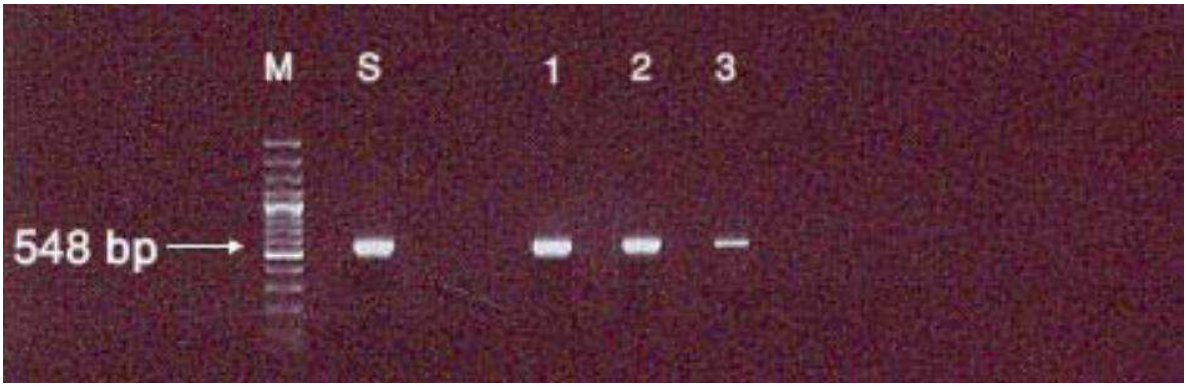

Figure 1: gel electrophoresis image of the PCR productions by means of specific primer for mycobacterium. 548bp bonds are observable in 3 positive samples

Then, the negative – mycobacterium samples were examined for mycoplasma infection. After observing the 465-bp bond in agarose gel, genus positive samples were confirmed and gone under PCR reaction for *mycoplasma Pneumoniae* species identification. 139-bp bonds on agarose gel show the positive *mycoplasma Pneumoniae.* (figure 2,3)

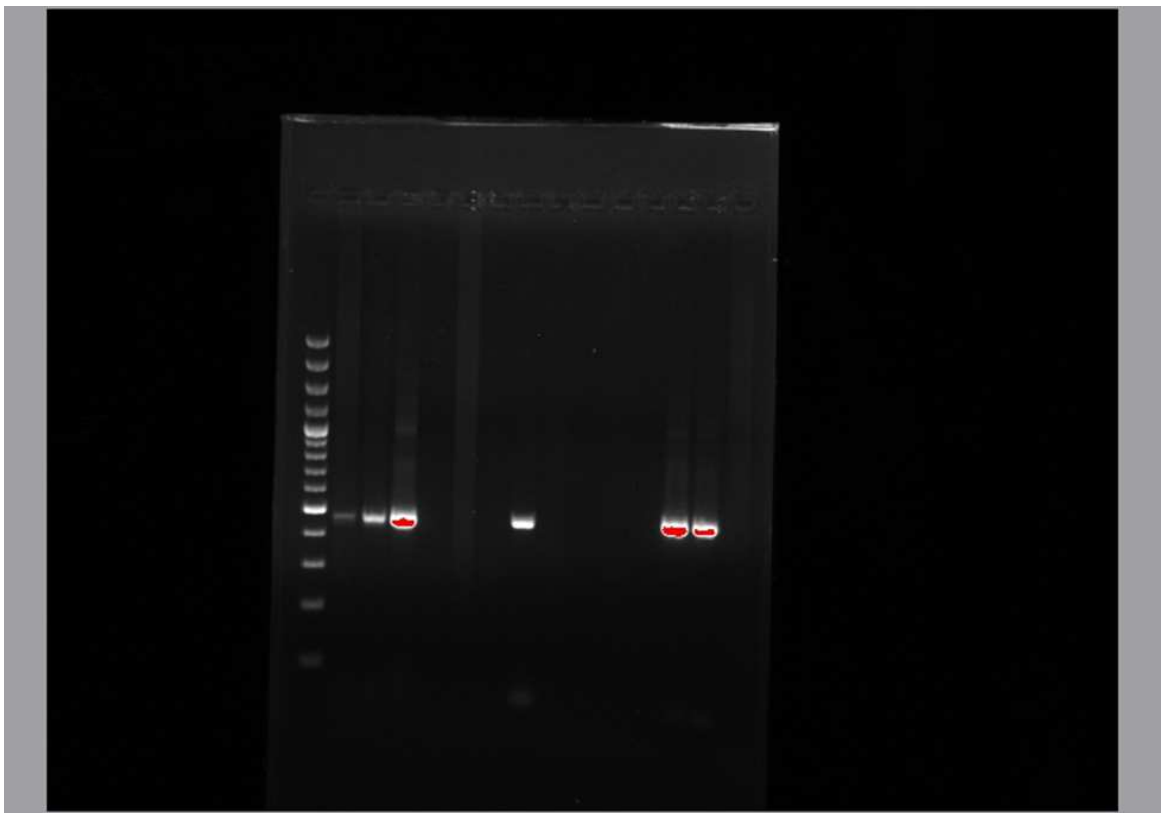

Figure 2: the gel electrophoresis of PCR production by means of specific primer for mycoplasma. 465bp bonds are observable in 3 positive samples

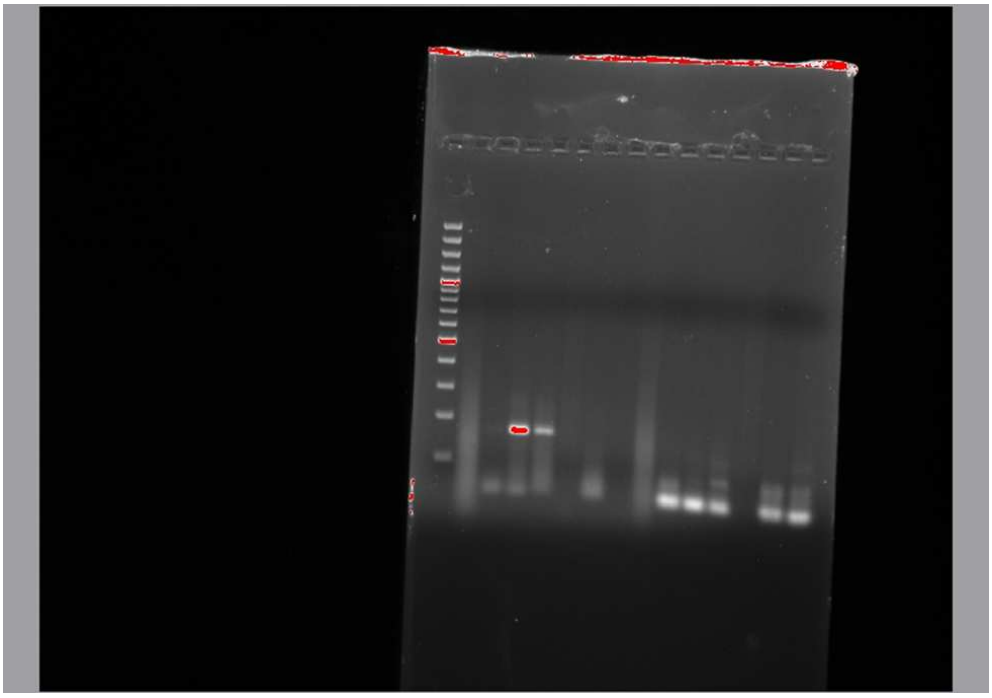

Figure3: the gel electrophoresis of PCR production by means of specific primer for *mycoplasma Pneumoniae*. 139bp bonds are observable in 3 positive samples

The results of PCR for mycoplasma in Table 4 shows that, in 18 out of 50 received sputum samples, mycoplasma genus has been confirmed. Also, by means of PCR, 12 positive sample of *mycoplasma Pneumoniae* has been observed.

PCR for species			RPCR for genus		
Total percent	Percent	Number	Percent	Number	
24	66	12	36	18	**Positive**
76	34	6	64	32	**Negative**
100	100	18	100	50	**Total**

DISCUSSION

The current study has been done for the first time in Iran, shows the amount of infection to pulmonary mycoplasma in patients who show tuberculosis symptoms but do not have mycobacterium infections. Also, the amount of infection to in these patients has been determined. For the first time in Kerman, PCR technique was used for isolation of pulmonary mycoplasma and the feasibility for continuing the rest of work as a common experiment in identifying the important factors of chronic pulmonary infections has been provided for future reference. The outbreak of chronic pulmonary infections is severe in the World and not only does it bring sanitary problems, but also causes economic problems related to long and high treatment costs and long absence of the working staff in the society. (14, 15).tuberculosis is one of the diseases that involve the pulmonary system chronically. Tuberculosis is a contagious disease. The related bacilli to this disease is *mycobacterium Tuberculosis* or Koch's bacillus because Robert Koch, the German professor, discovered it at 1882 A.D. one of the subcategories of this disease is called bovine tuberculosis and it is common between human and livestock. This disease is of hygienic problems in underdeveloped countries. (15). Lungs are usually the victims in tuberculosis. Researches in Iran show that only 10 percent of the patients which tuberculosis symptoms who are referred to laboratory by specialists, are mycobacterium positive and therefore another factor has caused chronic and severe symptoms, which, bacterial factors are of utter importance among the causes.(3). In his study therefore, it has been tried to isolate and introduce the nature of *mycoplasma Pneominiae* from these patients. Despite of various researches related to isolation of *mycoplasma Pneominiae*, infection examination regarding to this bacterium has been never done by this attitude (3,16,17). Thus, PCR test was conducted on the samples of patients who were suspicious to tuberculosis, in order to confirming the mycobacterium infection and then, looking for mycoplasma began in patients who were

mycobacterium negative but showed clinical symptoms of tuberculosis such as chronic coughs with bloody sputum, fever, Sleep hyperhidrosis (night sweats) and weight loss. One of the problems regarding to tuberculosis diagnosis is that some of its techniques are too time-consuming, e.g. sputum culture needs 4 to 12 weeks. (1,12,15). Furthermore, isolation of *mycoplasma Pneominiae* shows poor results and therefore, using molecular techniques is in priority. (18). Similar studies which used culturing technique accompanied with other techniques such as PCR show that culturing technique is time-consuming and it has too many false negative results. (19)

Mycobacterium genus has been detected in this study and we did not pay attention to any specific species in detail, because various species of this bacteria can be contaminating and cause pulmonary tuberculosis symptoms.(1). No previous studies related to identification of genus and species of the pathogen of chronic pulmonary infection disease by means of PCR and determination of its molecular nature (mycobacterial and/ or mycoplasma infections) have been conducted in Kerman before. in spite of the endeavours regarding to prevention and control of this disease, without accurate identification of the pathogens, treatments and policymaking will be practically useless.

Results of this study show that more than one third of the patients who are suspicious to tuberculosis but do not have the disease, are mycoplasma positive, which regarding to the 90%-demographic studies (i.e. mycobacterium negative patients who show tuberculosis symptoms), shows a high breakout of mycoplasma infection in chronic pulmonary infections. Of course, as there has been no researches related to specific climate of Kerman and its correlation to mycobacterial infection and its outbreak in patients with chronic pulmonary disease, further studies are needed before assessment, because climate affects the rate of coming down with tuberculosis. (12)

mycoplasma Pneominiae has been isolated from More than two third of patients infected with pulmonary mycoplasma. This shows the importance of this genus among other species in chronic pulmonary infections, but, there might be the possibility of multiple and simultaneous infections by various species and further studies regarding to this issue are required. Previous works show the high rate of chronic infections by some of bacteria including *mycoplasma Pneominiae* that can have the direct pathogenesis on myocardium, cause inflation on coronary artery and rupture in thrombosis and atheroma in above-mentioned artery. A meaningful relation between these two parameters in patients with myocardial backgrounds and lack of a meaningful relation between them in control group can be another reason for the interference of chronic infections in development of atherosclerosis and exacerbation of myocardial ischemia (17). Therefore, it can be inferred that lungs' health care against mycoplasma infection is amongst the important factors that should be taken into account in society health programme and its regular pursuit for prevention and identification of these infections is of utter importance. Also, fast and accurate identification is one of the factors that decreases the cost of infectious diseases. Traditional culture-based methods are still considered as golden standard in some cases, but they generally do not have the characteristics of a desirable method for identification of microorganism, including *mycoplasma Pneominiae*. (8, 9)

Conclusion

The results of this study show that other bacteria, especially mycoplasma have a significant role in Non-tuberculous chronic pulmonary infections. As laboratory tests are only conducted for identification of tuberculosis's infectious factors in suspicious to tuberculosis cases and by observing the negative results, complimentary tests are not performed for other infectious factors (15), therefore, identification test for mycoplasma is suggested. Also, as there is a possibility for simultaneous mycobacterium and mycoplasma pulmonary infection in tuberculosis patients, a separate study regarding to investigating these infections seems unavoidable. Results show that in more than two third of mycoplasma chronic pulmonary infections, *mycoplasma Pneominiae* genus takes part. Finding other affecting species in infections are helpful in identifying their factors.

Acknowledgement

This study, is the result of master-of-science thesis in Kerman's Azad university science and research branch and we hereby would like to thank the Vice Chancellor for Research and Technology of the University. Also, we would like to express our sincere gratitude to Ms. Sahar Amirpour for technical counseling.

REFERENCES

1. Ling Lin P, Flynn JL. Understanding Latent Tuberculosis: A Moving Target. J Immunology. 2010;1(185):15-22.
2. Ray S, Talukdar A, Sonthalia N. Diagnosis and management of miliary tuberculosis: current state and future perspectives. Therap and Clin Risk Management. 2013;9:9-26.

3. Moosavian SM, Pordeli HR. Survey of Respiratory and Urogenital Infections Due to Mycoplasma in the Hospitalized Patients in Ahwaz Imam Khomeini Hospital. Med J Kerman Med Sci Uni. 2004;4(10):185-192.[Persian]
4. Calvillo–King L, Arnold D, Eubank KJ, Lo M, Yunyongying P, Stieglitz H, Ethan A, Halm EA. Impact of Social Factors on Risk of Readmission or Mortality in Pneumonia and Heart Failure: Systematic Review. J General Int Med. 2013;28(2):269-282.
5. Okazaki N, Ohya H. *Mycoplasma pneumoniae* isolated from patients with respiratory infection in Kanagava prefecture in 1976-2006: Emergence of macrolide-resistant strains. Jpn J Infect Dis. 2007;60: 325-326.
6. Klompas M, Kleinman K, Khan Y, Evans RS, Lloyd JF, Stevenson K, Samore M, Platt R. Rapid and Reproducible Surveillance for Ventilator-Associated Pneumonia. Clin Infec Dis. 2012;54(3):370-377.
7. Diaz MH, Winchell JM. Detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* directly from respiratory clinical specimens using a rapid real-time polymerase chain reaction assay. Diagn Microbiol Infect Dis. 2012;73(3):278-280.
8. Kumar S, Saigal SR, Sethi GR. Rapid diagnosis of *Mycoplasma pneumoniae* by polymerase chain reaction in community-acquired lower respiratory tract infections. Trop. Doct. 2011;41(3):160-162.
9. Rostamizadeh V, Pourbakhsh SA, Asli E, Hadadi A. Detection of Mycoplasma salivarium Contamination in Cell Culture using PCR Method. Modares J Med Sci: Pathobiology. 2013;16(3):109-108.
10. Marcelo GM, Galit ZI, Dennis S, Ben TA, Ben SL, Shimon R, Itay A. The Role of Physical Examination in Establishing the Diagnosis of Pneumonia. Pediatric Emergency Care. 2013;29(8):893–896.
11. Chaudhry R, Tabassum I, Kapoor L. A fulminant case of acute respiratory distress syndrome associated with *Mycoplasma pneumoniae* infection. Indian J Pathol Microbiol. 2010;53(3): 561-563.
12. Yu X, Wen Z, Chen G, Li R, Ding B, Yao Y, Li Y. Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated from South-central in China. J Nature Pub. 2013;17(5):122-133.
13. Kumar S, Saigal SR. Rapid diagnosis of *Mycoplasma pneumoniae* by polymerase chain reaction in community-acquired lower respiratory tract infections. Tropical doctor. 2011;41(3):160-162.
14. Chen Z, Ji W, Wang Y, Yan Y, Zhu H, Shao X, Xu J. Epidemiology and associations with climatic conditions of Mycoplasma pneumoniae and Chlamydophila pneumoniae infections among Chinese children hospitalized with acute respiratory infections. Italian J of Pediatrics. 2013;39(1):34-39.
15. de Vries G, Aldridge RW, Caylà JA, Haas WH, Sandgren A, van Hest NA, Abubakar I. Epidemiology of tuberculosis in big cities of the European Union and European Economic Area countries. Surveillance and outbreak rep. 2014;19(9):726-732.
16. Wu PS, Chang LY. Epidemiology and clinical manifestations of children with macrolide-resistant *Mycoplasma pneumoniae* pneumonia in Taiwan. Pediatric Pulmonology. 2013;34(6):345-356.
17. Honarmand H, Taghizadeh S, Rahbar-Taroimsa B. Comparison of seropositivity rate of *Mycoplasma pneumoniae* in patients with acute myocardial infarction and without it. Sci J Ilam Uni Med Sci.2011;14(3):57-63.
18. Han X, Li S. Amplification of 16S rDNA by nested PCR for measurement of *Mycoplasma pneumoniae* DNA over time: clinical application. J med microbial. 2012;61(3):426-430.
19. Chalker V, Stocki T. *Mycoplasma pneumoniae* infection in primary care investigated by real-time PCR in England and Wales. Euro J Clin Microbiol Infec Dis. 2011;30(7):915-921.