Advanced and conventional Molecular techniques in the diagnosis of *Mycoplasma pneumoniae* in patients with pneumoniae

Muntaha M.Hassan[1], Mushtak T.S. Al-Ouqaili [2], Nidhal A.Mohammed [3]
[1]College of Medicine - University of Anbar.
[2]Dean of College of Pharmacy- University of Anbar.
[3]College of Medicine-AL-Nahrain University.

Abstract:

Background:-*M. pneumoniae* is an important human pathogen that produces community-acquired respiratory tract infection. Diagnosis of *M. pneumoniae* infection is challenging and crucial for the timely initiation of the effective antibiotic therapy.
Objective: This study has been undertaken to detect *M. pneumoniae* in respiratory samples (throat swabs, throat wash and sputum) in patients with respiratory tract infection qualitatively by conventional polymerase chain reaction (PCR). Also, more advanced one, real time PCR was used to determine mycoplasmal target gene qualitatively and quantitatively.
Patients and methods: The study was performed on Seventy-five patients and thirty healthy subjects as control. Human genomic DNA was extracted and *M. pneumoniae* target gene (lipoprotein gene) was amplified using conventional PCR. Negative, positive controls and internal controls were involved in each experimental run. The amplified products were analyzed in 2% agarose gel and visualized using Red safe staining. In real time PCR, specific primer and probe mix depending on TaqMan® principle was used to detect P1 adhesion gene through FAM channel. A fluorogenic probe was included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification. Data were analyzed using Smart-cycler software and *M. pneumoniae* DNA copy number was estimated from the cross point threshold relative to positive standard.
Results: Thirty five patients (45.5%) were positive by PCR and Thirty two (42.6 %) were positive by Real-time PCR. The highest rate of infection by using two molecular methods was of less than 20 years of age. The quantity of *M. pneumoniae* DNA target gene in positive Real-time PCR was ranged between 10-2000copies/µl.
Conclusion: The study concluded that both of molecular techniques conventional and real-time PCR are a rapid, reliable and ideal in diagnosis of *M. pneumoniae* using throat swabs, throat wash and sputum samples.
Key word: *Mycoplasma pneumoniae*, PCR, Real-time PCR

Corresponding author: Muntaha M.Hassan, E-mail: mun_alouci@yahoo.com

Introduction:

M. pneumoniae is an important human pathogen[1] that produces community-acquired respiratory tract infection, such as upper respiratory inflammation, bronchitis and pneumonia[2] and has been associated with acute exacerbation of asthma[3].

This pathogen is also responsible for producing a wide spectrum of non-pulmonary manifestation including neurologic, hepatic, cardiac disease, hemolytic anemia, polyarthritis and erythema multiformis[4].

M. pneumoniae is the smallest and simplest self-limiting bacteria[5]. It is classified in the class of mollicutes (soft skin). It is characterized by the lack of cell wall and the small amount of genetic materials that comprising the genome of this organism[6].Diagnosis of *M. pneumoniae* infection is challenging because of the small size of this organism about 0.3µl in diameter, the absence of cell wall consequently, stains poorly with gram stain and pleomorphoses, the fastidious nature of this pathogen and the transient asymptomatic carriage[4]. In addition to the difficulty in sampling of the lower respiratory tract infection (LRTI).

Currently available methods for diagnosis of *M. pneumoniae* infection have their limitation for example cold agglutination test is simple to perform but it is not very reliable indicator of *M. pneumonia* infection as it is elevated in 50-60% patients[4]. It is also elevated with various other infectious agents for example *Epstein-Barr virus*, *Cytomegalovirus*, *Klebsiella pneumoniae*, *Leishmania* sp. as well as in the course of malignancies and auto-immune diseases[7]. Complement fixation test which measures the early IgM response has its limitations such as low sensitivity and specificity because glycolipid antigen mixture used may be found in other microorganisms, as well as human tissues. Culture is laborious, expensive, lack of sensitivity and time-consuming [(1,8)].Serological diagnosis need acute and convalescent-phase specimens and many adult patients do not produce IgM antibodies upon re-infection with *M. pneumoniae* due to previous exposure to the organism.

Owing to the previous reasons, Polymerase chain reaction (PCR) has gained considerable interest in the diagnosis of *M. pneumoniae*, molecular methods have the potential to produce rapid, sensitive and specific results in addition to the broad spectrum of specimens suitable for the detection of this pathogen in respiratory infection[9]. Recently developed 5'nuclease and real-time PCR formats, allowing automated PCR amplification and detection of *M. pneumoniae* and other pathogens. This system is highly speed, less handling of PCR products and decreased risk of false-positive results due to carryover contamination [10].

Several regions in the *M. pneumoniae* genome have been used to detect and identify this pathogen by PCR and other molecular techniques such as P1 adhesin gene, species specific 16S rRNA, the mycoplasmal ATPase, operon gene and the tuf gene [6]. Thus, this study has laid down to detect *M. pneumoniae* in respiratory samples (throat swabs, throat wash and sputum) in patients with respiratory tract infection qualitatively by conventional polymerase chain reaction (PCR). Also, more advanced one, real time PCR was used to determine mycoplasmal target gene qualitatively and quantitatively.

Patients and methods

Patients:- Seventy five patient with pneumoniae were selected from the department of Internal Medicine of the AL-Ramadi teaching hospital depending on X-ray, signs and symptoms by the physicians from November 2009 to April 2010, they were investigated for microbiological diagnosis based on respiratory samples (throat swabs, throat wash and sputum). Thirty healthy person with the same socioeconomic standard and age groups were evaluated as control group. All cases and controls were subjected to full history including the period of onset of the current illness, fever, cough, residence and occupation.

Samples processing: - In the laboratory, prior to DNA extraction, respiratory samples were processed as follows:

A-Sputum samples: - They were homogenized by adding an equals volume of mucolytic agent (2-mercaptoethanol 0.1M) and vortex vigorously. After that

incubation for 30 min at room temperature and vortexing was done. Then, the solution was centrifuged at 10000 g / min for 10 min and the supernatant was removed. The pellet was resuspended in 100 ml of saline water.

B-Throat swab:- The swabs were agitated for 30 min and squeezed against the walls of the tubes, the swabs were discarded and the suspension were centrifuged at 3000g/min for 10-15 min, the supernatant was removed and discarded, the pellet was re-suspended in 100ml of saline water and stored until further processing[11].

C-Throat wash:- Ten ml of throat wash were centrifuged at 3000g/min for 10-15 min, the supernatant was removed and discarded. Then, the pellet was responded in 100ml of saline water.

An aliquot of 100 µl of each of the specimens were taken and subjected to DNA extraction utilizing commercial kit (DNA sorb –B, sacace, Italy) using the blood and body fluid spin protocol .

DNA amplification by conventional PCR:-

Amplification was performed using specific *M. pneumoniae* primer (lipoprotein gene)[9]. The PCR was carried out in PCR mixture, ready to single use. In a total volume of 30 µl and the volume of the DNA sample was 10µl. The PCR mixture contain 10ml µl of PCR –mix-1 and 10µl of PCRmixture 2 and 10 µl of DNA sample, the test tubes were placed in a thermal-cycler (ESCO) and heated at 95 Cº for 5 min, after apre-denaturation, 42 cycles consisted of denaturation at 95Cº for 30 sec, annealing at 61 Cº for 30 sec, extension at 72Cº for 30 sec. and final extension after the last cycle. Strict protocols were established to prevent contamination, including isolation of PCR reagent preparation, PCR products detection, amplification site, aliquoting of reagents, all glassware and pipettes tips were decontaminated. Negative and positive controls were used in each experimental run[11]. Internal control was employed to determine the presence of inhibitory factors [12, 13]. The amplification products were analyzed in 2% agarose gel and visualized after staining with Red safe by ultraviolet transilluminator[9].

Real-time PCR:-Real-time PCR kit for *M. pneumoniae* detection was provided by specific primer and probe mix and this can be detected through the FAM channel. The primer and probe mix provided the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the *M. pneumoniae* DNA/cDNA. A fluorogenic probe was included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms. For copy number determination, the standard curve dilutions series were prepared as the kit instruction, detection mix was included the following components:(10 µl ofMasterMix,1 µl of *M. pneumoniae* Primer/Probe mix,4 µl of RNAse/DNAse free water), the final volume of 15 µl of this mixture was pipette in each well, then 5µl of each of diluted samples DNA template, negative control and standard template(positive control) were pipette into each appropriate well.

The amplification protocol included the following steps:- (Enzymatic activation 95Cº for 10 min, denaturation at 95Cº for10 sec, and data collection at 60 Cº for 60 sec.). The protocol included 50 cycles, each cycle consist of denaturation and data collection.

Data were analyzed using Smart-cycler software using arithmetic baseline adjustment, *M. pneumoniae* DNA copy number was estimated from the cross point threshold relative to positive standard. The standard curve correlates each copy number with a particular CT-threshold (Ct)

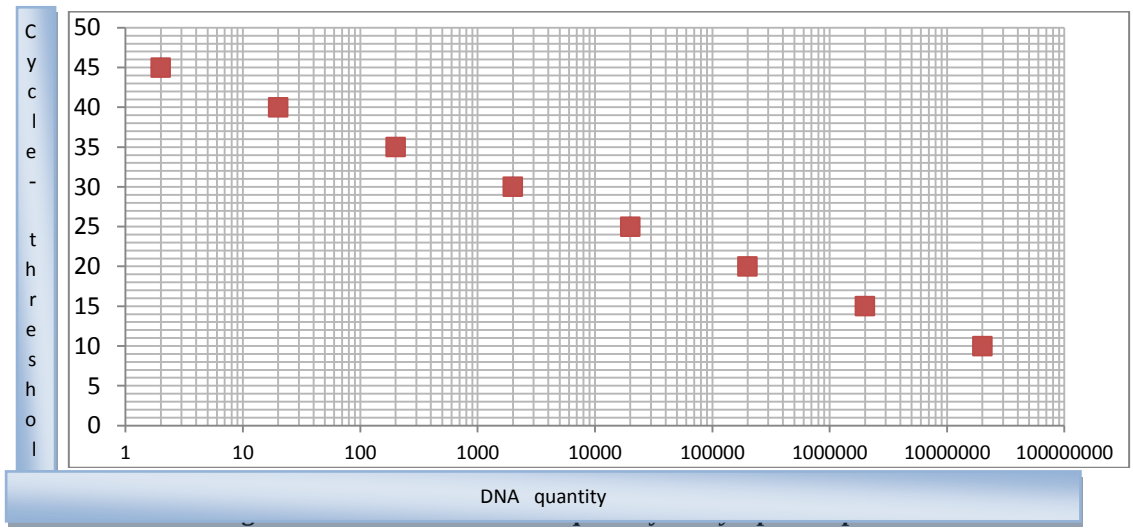

Fig1. The copy number values of the unknown samples are driven from the standard curve.

Results:-

The study was performed on 75 patients with pneumonia (40 males and 35 females) from different age group (1-80 years). Control subjects were 30 healthy subjects. In this study, Thirty four samples (45.5%) were positive with PCR for *M. pneumoniae*. The highest infection rate(15/40) (20 %) was in the age group of (<20 years), followed by (14/18) (18.6%) for age group of (20-40 years) and (3/8) (4 %) for age group of (40-60) and the lowest rate was (2/9) (2.9 %) in (>60years) age group as shown in Fig2.

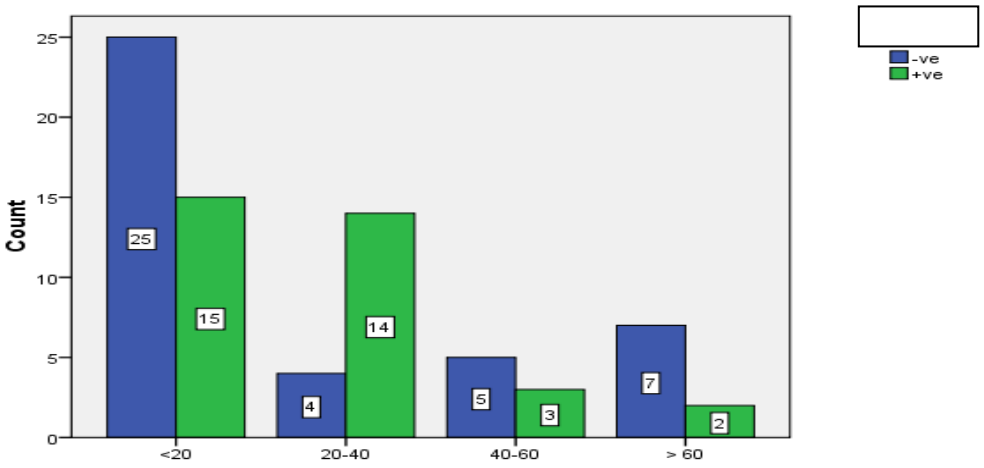

Fig. 2. The positive and negative PCR for *M. pneumoniae* in relation with age group.

Thirty four samples of seventy five (45.5%) were positive with PCR for *M. pneumoniae* in comparison with control subjects only 2 from 30 subject (6.6%) were positive with this test, Fig.3.

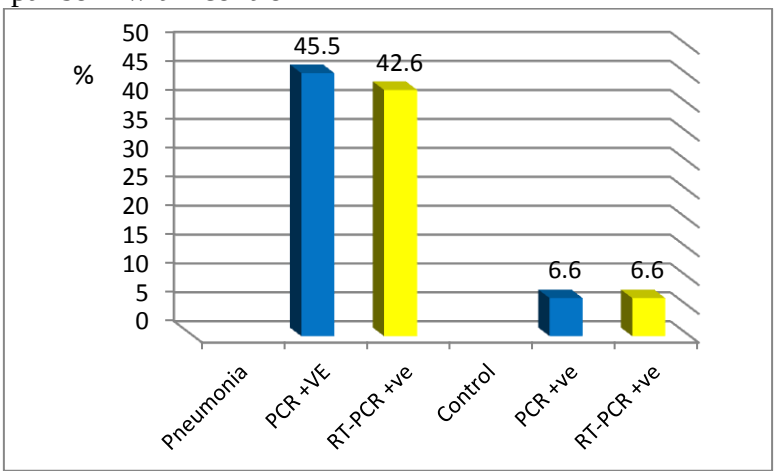

Fig.3. The comparison between pneumonia patients and control group in the results of PCR & RT-PCR used in the detection of *M. pneumoniae*.

The comparison between study and control group with PCR test by Z-test statistical analysis reveals that the difference was significant, where Z=3.815 and P value=<0.0001.

Thirty two samples of seventy five (42.6%) were positive with Real-time PCR, Fig.4 Shows the relation between positive and negative Real-time PCR for *Mycoplasma pneumonia* and the age groups; the highest rate (14/75)(18.8%) was for the group of (<20 years), (13/18) (17.2%) for group of (20-40 years), (3/8) (4%) for group of (40-60years) and (2/9) (2.6%) for age group of(> 60 years). The DNA copy number was estimated from the cross point threshold relative to positive standard (see figure 1).

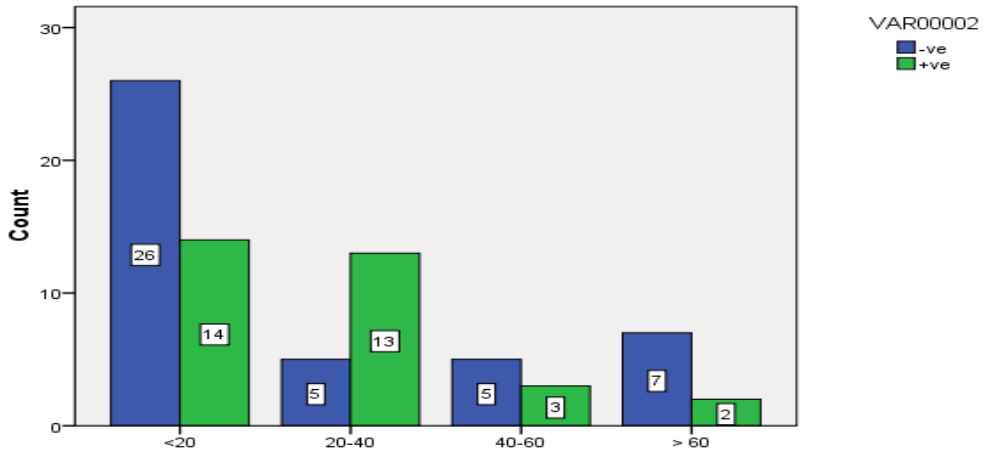

Fig.4. Distribution of Real-time PCR result for *M. pneumoniae* cases according to age group.

The comparison between study and control groups with Real-time PCR test was analyzed by Z –test, results shows that Z=3.58,P value <0.0001, which means that the difference between two groups was significant, 42.6% from study group were

Mycoplasma pneumonia positive, while only 6.6% from control group were *Mycoplasma pneumonia* positive, Fig.3. The comparison between two molecular methods PCR and Real-time PCR for *Mycoplasma pneumonia* diagnosis shows that results were approximate and the difference between tow molecular diagnostic methods was not significant as represented in figure 5.

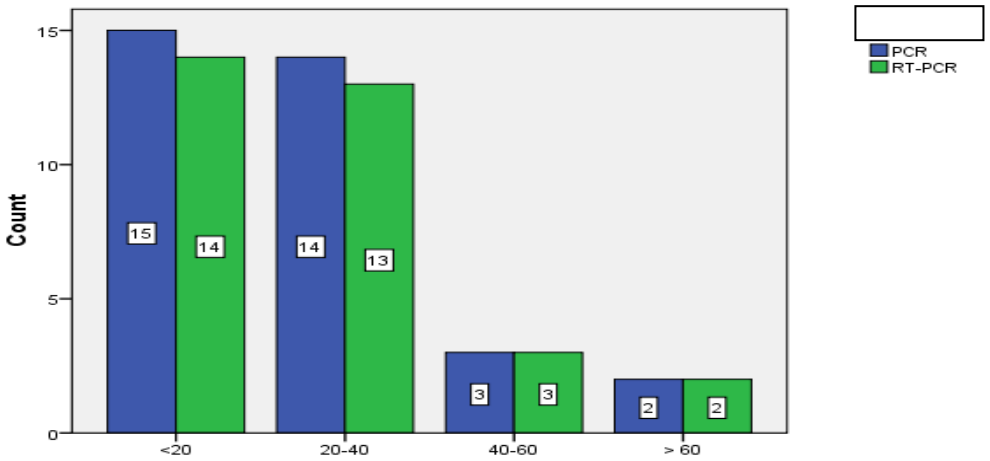

Fig.5. The comparison between positive conventional PCR and Real-time PCR for *Mycoplasma pneumoniae* in relation with the different age group.

The amplified gene P1 adhesin was stained with Red safe stain and visualized on agarose gel 2% by electrophoresis. The length of specific amplified DNA fragments of internal control used in this study was 565bp while for *M. pneumoniae* was 325 bp.Fig.6.

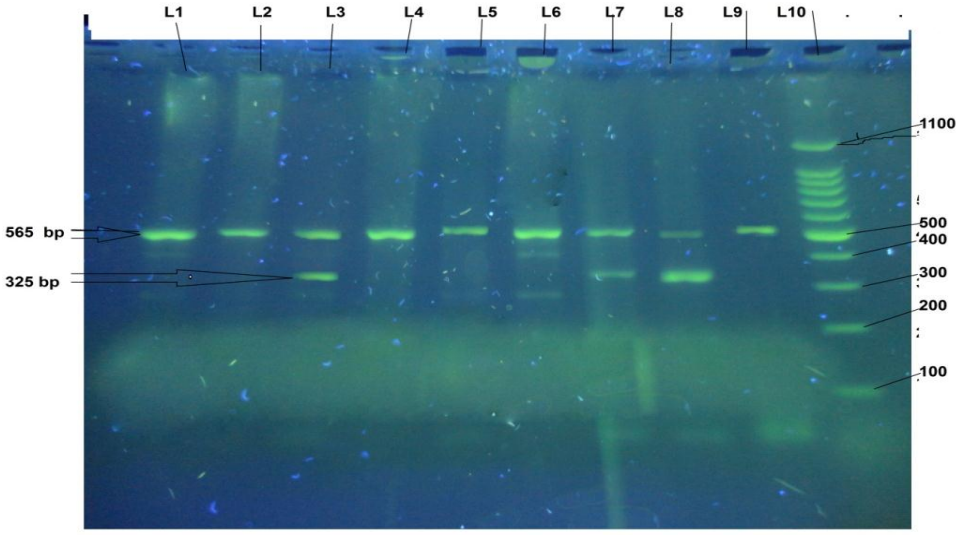

Fig6. Agarose gel electrophoresis(2%) with Redsafe stain. Bands with amplified P1 adhesin gene obtained from respiratory samples, the positive results were (L3 and Ll7) while (L1,L2,L4,L5 and L6) were negative results. Positive and negative controls were represented in L8 and L9 respectively. The internal control was positive in all samples and controls. Bench top PCR marker as DNA molecular weight marker with (100-1100 bp)on the right.

Discussion

It is well realized that since it is impossible to identify a *M .pneumoniae* infection solely on the basis of clinical signs and symptoms, the correct diagnosis of *M. pneumoniae* infections is so important to allow the appropriate antibiotic treatment of such patients.

Lab diagnosis of *M. pneumoniae* greatly hampered by the lack of the standardized, sensitive and specific methods for the detection of this pathogen[9]. *M. pneumoniae* culture can often take several weeks, requires special media and expertise, and is insensitive and prone to contaminants and inhibitors. Serological assays such as complement fixation and commercially available immunoglobulin detection kits are by nature of retrospective, requiring paired serum samples from both acute and convalescent phases and provide questionable specificity and sensitivity results[14]. Owing to these limitations of the conventional assays for the detection of *M. pneumoniae*. This study was applied the molecular methods PCR and qPCR (Real-time PCR) to detect *M. pneumoniae* nucleic acids (DNA). The first character of the molecular methods is the wide spectrum of the samples suitable for *Mycoplasma pneumonia* DNA detection[11]. Many studies used different clinical samples to be used in molecular field like sputum, nasopharyngeal aspirates and throat swabs[11], bronchioalveolar lavage[8], nasopharyngeal and pharyngeal exudates[2]. In this study, sputum, throat wash and throat swabs were used according to the patient's age and situation. The second character is the different genes can be used as a primer in detection of *M. pneumoniae* DNA, the most frequent genes used as target are P1 adhesin,16 rRNA and the gene coding elongation factor (tu16)[9].

The choice of genomic region to be amplified will determine the specificity of detection from the outset, the sensitivity of the detection assay is connected with the nature of the target region via the efficiency of primer binding which determines the efficiency of amplification. Optimal primer length varies between 18-24 nucleotides and the length of PCR products has an inverse correlation to the efficiency of amplification, relatively short targets do not only facilitate high sensitivity of detection but are also preferable for quantitative PCR assays[15].

In this study two target regions were used. The P1 adhesin gene in conventional PCR, which described by Ieven and associates 1996 and Ursi and co-workers 1992[16, 17], and 16s rRNA in Real-time PCR which mentioned by van Kuppeveld 1994[18].

De Barberyrac et al 1993 showed that PCR of fragment of the P1 gene or 16s rRNA gene was to be considerably more sensitive than culture for detection of *M. pneumoniae*[19].

It is essential that respiratory samples inhibitory to PCR and real-time PCR are identified to ensure that they are not falsely reported as negative ,amplification inhibitors occurs frequently and may be difficult to eliminate, such as heme compounds, polysaccharide in sputum, mucolytic agent added to sputum and some reagents. Different type of internal control can be used to discriminate between a false –negative reaction and truly nonreactive sample[9].When performing DNA extraction; it is often advantageous to have an exogenous source of DNA template. This control DNA was co-purified with the sample DNA and was detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration. Analysis of PCR results is based on the presence or absence of specific bands of amplified DNA in agarose gel (2%), the length of specific amplified DNA fragments of internal control used in this study was 565bp while for *M. pneumoniae* was 325 bp, in this study the successful purification and amplification was guaranteed by the visualization of internal control bands with each sample negative or positive for *M. pneumoniae*. In real time PCR the successful of DNA purification and amplification was guaranteed by the detection of I.C. through the VIC channel and gives a CT value of 26 +/-3, where a separate primer and probe mix are supplied

with this kit to detect the exogenous DNA using real-time PCR apparatus, the primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the *M. pneumoniae* target DNA which detected through FAM channel, even when present at low copy number

PCR results showed that infection with *M. pneumoniae* was 45.5% (54 from 75) which include all age group which ranged between (1-80 years), older studies reported M. *pneumoniae* to be somewhat uncommon in children aged less than five years[20], while the later studies have documented that *M. pneumoniae* may occurs endemically and occasionally epidemically in older patients as well as children under five years old[21], which agree with our study results.

In accordance with our results, Raty showed positive results in PCR as follows: 69% in sputum samples and 50%of the aspirates and 37.5% of the swabs[11], while Honda and associates showed that PCR was positive in 20.9% of specimen[22]. Ursi and Dorigo concluded that PCR results could be added to the criteria for the diagnosis of *M. pneumoniae* infections and could even replace culture [17, 23].

Real-time PCR kit which used in this study detect 2 copies up to 2000000 copies of the target sequence in the 5µl samples used in the assay, sample with copy number less than the lowest standard in the assay were deemed negative. In accordance with our results 42% were considered positive by Real-time PCR), the Ct value of 31±3 was within the normal range, Winchell and co-workers, demonstrated that 18 of 35 pneumonia cases (51%) were *M. pneumoniae* positive, the Ct values ranged between (26-35)[14]. Thurman et al showed that 21% yielded positive results with real-time PCR, whereas in control group only 1.8% yielded positive results[24], while Chalker et al 2011 showed that the level of *M. pneumoniae* infection in qPCR based community surveillance of patients in England and Wales was low (1.7%)[25]. Pitcher and associates (2005) showed that out of 175 study pneumonic patients, 20(11.4%) were positive in real-time PCR assay[26]. Real-time PCR has an important advantage than the other diagnostic methods, it can detect the low level of colonization in patients with or without symptoms which may represent low levels of infection .

It is difficult to compare the results of PCR study and draw a single, comprehensive approach for reliable detection, because of the range of variables within each study such as specimen's types, nucleic acid extraction and amplification procedures, target selection and definition used in calculating data [14].

The positive results for *M. pneumoniae* DNA with two molecular diagnostic methods (PCR and Real-time PCR) for control group were (6.6%), this result may be belong to asymptomatic carriage or prior infections or they had exposure to case patients (they were household contact or they attend the same place; schools and businesses). Asymptomatic carriage has been reported to occur within the community and up to 13% of healthy adults, they may act as a carrier for *M. pneumoniae*, particularly during epidemic periods because *M. pneumoniae* spreads efficiently within household and close living quarters with incubation period as long as three weeks [14].

The study concluded that *M. pneumoniae* was the etiological agent of about (42-45.5)% of community acquired pneumonia by using conventional PCR and Real-time PCR, these molecular techniques are a rapid; they are suitable for same day diagnosis of *M. pneumoniae* and batch processing of respiratory samples for clinical screening, reliable and ideal in diagnosis of *M. pneumoniae*.

Acknowledgements:-

All authors wish to extend their gratitude to Dr. Omer A. Mohammed at the Department of Asthma and allergy, Ramadi Teaching Hospital for his assistance in the study by his medical comments.

References:

1. Waites, K.B. and Talkington, D.F. *Mycoplasma pneumonia* and its role as a human pathogen.Clin. Microbiol. rev. 2004 ; 17(4):697-728.
2. Huong, P.L.T, Ngo, T.T., Nguyet, N.T.T, Van, T.K., Hang, D.T., Huong, V.T.T., Anb, D.D. and Sasaki, T. First Report on Clinical features of *Mycoplasma pneumoniae* Infections in Vietnamese Children. Jpn.J. Infect. Dis.2007; 60:370-373.
3. Marie, M.A.M. Association of *Mycoplasma pneumoniae* in asthma pathogenesis. Tuber. Respir. diseas. 2008 ; 65(4):261-268.
4. Kashyap, S. and Sarkar, M. *Mycoplasma pneumonia*: Clinical features and management. Lung India .2010 ; 27(2):75-85.
5. Levinson, W. Review of medical microbiology and immunology.2008; 10th edition .Mc Graw Hill. 344-349.
6. Winn, W.C., Allen, S.D., Janda, W.M., Koneman, E.W. Procop, G.W., Schreckenberger, P.C. and Wood, G.L. Color Atlas and Textbook of Diagnostic Microbiology .2006 ; 6th ed .Lippincott Williams &Wilkins. U.S.A. 1043-1044.
7. Daxboeck, F., Krause, R. and Wenisch, C. Laboratory diagnosis of *Mycoplasma pneumoni*ae infection. Clin.Microbial.infect.2003; 9:263-273.
8. Touati, A., Bernard, A., Hassen, A.B. Be'be'ar, C.M. and Pereyre, S. Evaluation of five commercial Real-Time PCR for detection of *Mycoplasma pneumonia* in Respiratory Tract Infection.J.clin. Microbiol.2009; 47(7):2269-2271.
9. Loens, K., Ursi, D., Goossens, H. and Ieven, M. Molecular Diagnosis of *Mycoplasma pneumonia* Respiratory Tract Infections.J.Clin.Microbiol.2003; 41(11):4915-4923.
10. Hardegger, D., Nadal, D., Bossart, W. ,Altwegg, M. and Dutly, F. Rapid detection of *Mycoplasma pneumoniae* in clinical samples by real-time PCR.J.Microbiol.Methods.2000 ; 41:45-51.
11. Raty, R., Esa, R. and Kleemola, M. Sample type is crucial to the diagnosis of *Mycoplasma pneumonia* by PCR. J. Med. Microbiol. 2005 ; 54:287-291.
12. Kraft, M., Cassell, G.H., Henson, J.E., Watson, H., Williamson, J., Marmion, B.P., Gaydos, C.A. and Martin, R.J. Detection of *Mycoplasma pneumoniae* in the airways of adults with chronic asthma. Am.J.Respir.Crit.Care. Med.1998 ; 158(3):998-1001.
13. Loenes, K., Ursi, D., Ieven, M., and van Aarle, P. Detection of *Mycoplasma pneumoniae* in spiked clinical samples by nucleic acid sequence – based amplification. J.Clin. Microbiol. 2002 ; 40(4):1339-1345.
14. Winchell, J.M., Thurman, K.A., Mitchell, S.L., Thacker, W.L., and Fields, B.S. Evaluation of three real-time PCR assay for detection of *Mycoplasma pneumoniae* in an outbreak investigation .J.Clin. Microbiol. 2008 ; 46(9):3116-3118 .
15. Toouli, C.D., Turner, D., Grist, S. A., and Morley, A. A. The effect of cycle number and target size on polymerase chain reaction amplification of polymorphic repetitive sequences. *Anal. Biochem.* 1999; 280, 324–326.
16. Ieven, M., Ursi, D., Van Bever, Quint, H. W., Niesters, H.G.M. and Goossens, H. Detection of *Mycoplasma pneumoniae* by tow polymerase chain reaction and role of *M. pneumoniae* in acute respiratory tract infection in pediatric patient's .J.Infec.Dis.1996.173:1445-1452.
17. Ursi, J.P., Ursi, D., Ieven, M. and Pattyn, S.R. Utility of an internal control for the polymerase chain reaction . APMIS.1992 ; 100:635-639.
18. Van Kuppeveld, F.J.M., Johansson, K.E., Galama, J.M., Kissing,J., Bolske, G.and Melcher, W.J.16S rRNA based polymerase chain reaction compared with culture and serological methods for diagnosis of *Mycoplasma pneumoniae*. Eur.J.Clin.Microbiol.Infect.Dis.1994.13:401-405 .
19. De Barbeyrac, B., Barnet–Poggi, C., Febrer, F., Renaudin, H. Detection of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* in clinical samples by polymerase chain reaction .Clin.Infet.Dis.1993.17(1):83-89 .
20. Foy, H.M., Grayston, J.T. and Kenny G,T. Epidemiology of *Mycoplasma pneumoniae* infection in families .*JAMA* .1966 ; 197:859-866.
21. 15-Kashyap,S. and Sarkar, M. *Mycoplasma pneumoniae*: Clinical features and management. Lung India .2010 ; 27(2):75-85.
22. Honda, J., Takafumi, Y., and Kusaba, M. Clinical use of capillary PCR to diagnose *Mycoplasma pneumoniae*. J Clin Microbiol 2000; 38:1382-1384.

23. Dorigo-zetsma, J.W., Zaat, S.A.J., Wertheim-van Dillen, P.M.E., Spanjaard, L., Rijntjens, J., VanWaveren, G., Jensen, J.S., Angulo, A.F., and Dankert. J. Comparison of PCR, culture and serological tests for diagnosis of *Mycoplasma pneumoniae* respiratory tract infection in children. J.Clin. Microbiol.1999; 37:14-17 .
24. Thurman,K.A.,Walter,N.D.,Schwartz,S.B.,Mitchell,S.L.,Dillon,M.T.,Baughman,A.L.,Deutscher,M.,Fulton,J.P.,Tongren,J.E.,Hicks,L.A.,and Winchell, J.M. Comparison of laboratory diagnostic procedures for detection of *Mycoplasma pneumoniae* in community outbreaks .Clin.infect.dis.2009; 48(9): 1244 – 1249.
25. Chalker, V.J., Stocki, T., Mentasti, M., Fleming, D., and Harrison, T.G. Increased incidence of *Mycoplasma pneumoniae* in England and Wales in 2010: Multilocus variable number tandem repeat analysis typing and macrolide susceptibility .Eurosurveillance.2011; 16(19):1-5 .
26. Pitcher, D., Chalker, V.J., Shepard, C., George, R.C. and Harrison, T.G. Real-time detection of *Mycoplasma pneumoniae* in respiratory samples with an internal processing control . J. Med. Microbiol. 2006; 55:149–155