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Molecular Detection of 16SrRNA of *Chlamydia pneumoinae* and specific IgE in Asthmatic Patients

Hatim A. Al-Sabhany[1], Shehab A. lafi[2], and Mushtaq T. S. Al-Oqayli[3]
1-Ministry of Health, Al-Anbar, Ramadi General Teaching Hospital.
2-Department College of Medicine Anbar University, West of IRAQ.
3-College of Pharmacy, Anbar University, West of IRAQ.
Shehab58@yahoo.com

ABSTRACT

Background: *Chlamydia pneumoniae* is an obligate intracellular organism and it is one of the common causes of upper respiratory tract infection. Microbes were blamed to trigger asthma in different mechanisms, one of these microbes is *Chlamydia pneumoniae*.

Aims of the study: This study was done to show the rate of *Chlamydia pneumoniae* infection in asthmatic patients through detection of 16SrRNA of *Chlamydia pneumoniae* in blood and throat wash of patients using PCR test.

Methods: One hundred (120) asthmatic patients from both sexes and different age groups (2-84 years) were included in this study. These patients were attending the Clinic of Allergy and Asthma in Ramadi General Teaching Hospital, during the period extended from January to March 2011. Thirty (30) healthy individuals from both sexes were selected randomly to be considered as negative control group.

Blood specimens (5ml) and throat wash were taken from each patient, were employed for the detection of 16SrRNA of *Chlamydia pneumoniae* using PCR test. Pooled sera from 70 patients were employed for detection of IgE specific for *Chlamydia pneumoniae* using ELIZA test.

Results:

PCR test results of Blood specimens:

Among of 70 tested sera from asthmatic patients from both attack and remittance, thirty five (35), (50%) of the tested specimens were showing positive PCR test. Among patients, adult females were showing more positive PCR results for 16s r RNA of *Chlamydia pneumoinae* in their blood specimens 22 females (10, at attack and 12 at remittance), while all tested specimens from control group individuals were showing negative PCR results. Out of (50) tested throat wash specimens, five (5), (10%) of them were showing positive PCR for 16s r RNA of *Chlamydia pneumoinae*. Four (4) (8o%) of them were from adult patients at attack status and all control group individuals were showing negative PCR results IgE Specific for *Chlamydia pneumoinae* antigen, out of (54) tested sera from asthmatic patients, 37 of them were showing positive ELISA test for IgE specific for *Chlamydia pneumoniae* antigen, Adult females (24, (64. 8) were showing higher IgE positive results than males ($P <$ o.o5). All tested sera (15) from control group individuals were showing negative IgE *Chlamydia pneumoniae* antigen. Positive correlation was found between the results of IgE specific for *Chlamydia pneumoniae* and PCR results for blood and throat wash specimens in both attack and remittance.

Conclusion: We can conclude from this study that *Chlamydia pneumoinae* is involved with asthma post infection to the lower respiratory tract and induction of allergy mediators like IgE in both sexes.

Keywords: Asthma, PCR, *Chlamydia pneumoniae*.

INTRODUCTION

Chlamydia pneumoniae is an obligate intracellular organism and it is one of the common causes of upper respiratory tract infection (Cunningham 1998, Han 2005, Kocabas 2008). Microbial infections showed an increased importance in asthma pathogenesis and exacerbation (Kraft 2000, Fernandez, 2001, Lafi, 2004, Sutherland *et al*., 2004, Kocabas, 2008). Many bacterial types are able to release histamine from human mast cells and basophiles in vitro, this was suggested to be as pathological mechanism in intrinsic asthma (Brada *et al*., 1996, Holt and Bjorkeston 1997, Fernandez 2001). *Chlamydia pneumoniae* is one of the organisms showed importance in asthma exacerbation (Cunningham *et al*. 1998, Zhang *et al*. 2000, Lafi *et al*. 2004, Kocabas 2008, Mitchell *et al*. 2009). Immunoglobulins against *Chlamydia pneumoniae* like IgM, IgG, IgA and specific IgE were found to be associated with asthma, theses markers took diagnostic importance in asthma triggering factors (Numazaki *et al*. 1996, Miyashita *et al*. 1998, Black P. N *et al*. 2000, lafi, S.A. *et al*. 2004, Zaitsu 2007, Madro and Laprise 2010).

Few reports were done in this category for asthmatic patients in Iraq, particularly West of Iraq, Al-Anbar Governorate so this study is devoted.

Patients and Methods:

One hundred (120) asthmatic patients from both sexes and different age groups (2-84 years) were included in this study. These patients were attending the Clinic of Allergy and Asthma in Ramadi General Teaching Hospital during the period extended from January to March 2011. Patients were examined by senior physician to follow up their affection, some patients were suffering from asthmatic attack while other at remission.

Thirty (30) healthy individuals from both sexes were selected randomly to be considered as negative control group, these individuals were examined in the same way of asthmatic individuals.

Specimen collection:

Blood specimens (5ml) were taken from each patient, (3 ml) of them were employed for serum pooling and (2ml) were used for DNA extraction soon. Pooled sera were kept frozen at -20 C to be employed for IgE Specific for *Chlamydia pneumoniae* estimation.

Throat wash sample was taken following (Cadman, H. 2010), each patient was advised to brush his teeth with clean brush and water then advised to do throat wash and gargle with sterile normal saline. Gargles throat wash water was divided into four aliquots then centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the precipitate was pooled and kept frozen at -20C to be used for DNA extraction and were employed for the isolation of 16SrRNA of *Chlamydia pneumoniae* using PCR test.

Detection of *Chlamydia pneumoniae* 16SrRNA Using PCR Nucleic acid extraction:

DNA Sorb -B, DNA extraction kit (Sacace Biotechnology, ITALY) was employed for DNA extraction from each blood and throat wash specimens of patients and control group. Purity test was done for each extracted DNA samples. DNA samples were kept frozen at -20 to be used for PCR running later. Extracted DNA of patients and control individuals were amplified at Molecular Biology Unit of Microbiology Department, College of Medicine using PCR test:

a- Conventional PCR System.

PCR Premix –Accupower (Master mix, Bioneer, Korea).

Forward primer (CPn A)----------for 16SrRNA

Reverse primer (CPn B)----------for 16SrRNA (Alpha - DNA Canada).
Running PCR Program of (cycles for minutes) was employed.
PCR result was analyzed using gel electrophoresis for amplified specimens of nucleic acid in contrast to standard Molecular weight marker, DNA ladder (Promega, USA), and Bench Top PCR marker (Promega USA).

The PCR cycles consisted of an initial denaturation for 5 min. at 94C followed by 50 cycles of denaturation at 94 C for 45 seconds, annealing at 55 C and extension at 72 C for 1 min. The final extension was for 10 min. at 72 C. The PCR products were visulised by agarose gel electrophoresis with redsafe dye. Results were calculated and analyzed using statistical methods.

Specific IgE detection:

Serum specimens from 70 patients were employed for detection of IgE specific for *Chlamydia pneumoniae* using ELIZA test using (Biotek ELISA system, Spain). Antigen of *Chlamydia pneumoniae* obtained from (virion/ serion GMBHI, germany). Antigen Disk impregnation method described by (Lafi 2004[11]) was used to prepare antigen disks for ELIZA test. Other required items for ELISA test (IgE conjugate, washing solution, stop solution etc.) were obtained from (Diagnostic Automation; INC, USA). ElISA test was done as Described by (Fernandez-Botran and Vetvicka, 2000).

RESULTS

1- Age and sex distribution of patients:

One hundred twenty (120) asthmatic patients from both sexes were included in this study, sixty seven (67) (55.87%) of them were females and fifty three (53) (44.2%) were males female to male ratio was 1.26. Forty nine (49) (40.8%) of patients were in attack while seventy one (71) (59.2%) were at remittance stage.

Seventeen 17(14.1%) children were included in this study within age group (1-17 years), eleven (11) (64.7%) were males and six (35.3%) were females. Thirty (30) intact individuals from both sexes were included in this study resembling control group seven (23.4%) children and twenty three (23) (76.6%) were adults, eleven (11) (36.4%) of them were males and nineteen (19) (63.4%) were females (Table-1 and Fig. 1).

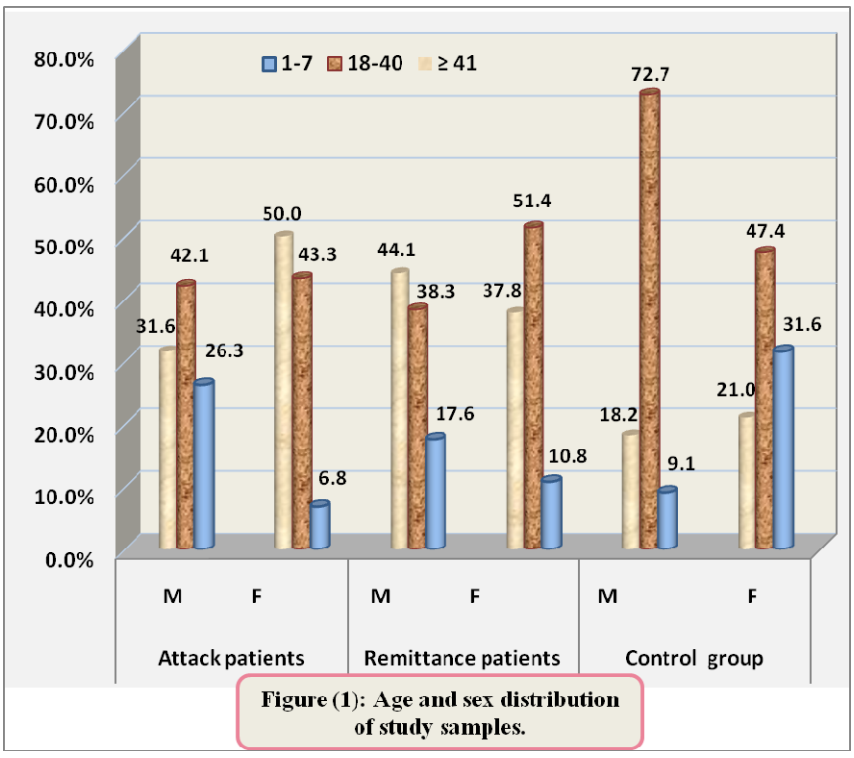

Figure (1): Age and sex distribution of study samples.

Table 1: Number and sex of the study patients and control group.

Age group	Patients group					Control group	
	Attack patients		Remittance patients				
	M No. (%)	F No. (%)	M No. (%)	F No. (%)		M No. (%)	F No. (%)
1 – 17 year	5 (26.3%)	2 (6.76%)	6 (17.6%)	4 (10.8%)		1 (9.1%)	6 (31.6%)
18 – 40 year	8 (42.1%)	13 (43.3%)	13 (38.3%)	19 (51.4%)		8 (72.7%)	9 (47.4%)
≥41 year	6 (31.6%)	15 (50%)	15 (44.1%)	14 (37.8%)		2 (18.2%)	4 (21%)
Total	19 (38.7%)	30 (61.3%)	34 (47.9%)	37 (52.1%)		11 (36.6%)	19 (63.%)
	49 (40.8%)		71 (59.2%)			30	
	120						

2- PCR test results:

A- Blood specimens:

Among of 70 tested blood sample from asthmatic patients from both attack and remittance stage, thirty five (35) (50%) of the tested specimens showed positive PCR test. Among patients, adult females showed more positive PCR results for 16SrRNA of *Chlamydia pneumoinae* in their blood specimens, 22 females (10 at attack and 12 at remittance), significant difference was found between them and males (P =0.001). Non significant difference (P= 0.61) was found between patients in attack (16 patients) and remittance status (19) patients. While all tested specimens from control group individuals showed negative PCR results, (Table 2 and Fig. 2).

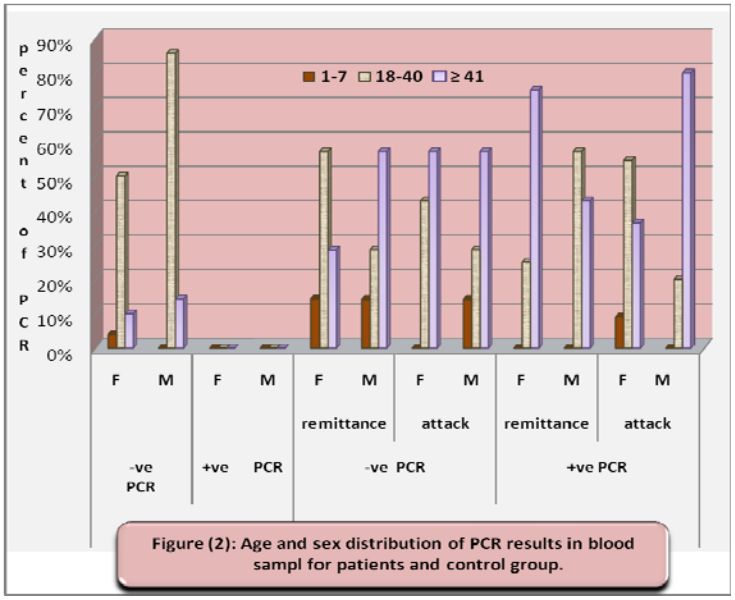

Figure (2): Age and sex distribution of PCR results in blood sampl for patients and control group.

Table 2: Age and sex distribution of PCR results in blood samples for patients and control group.

Age group	patients								Control			
	+ve PCR				-ve PCR				+ve PCR		-ve PCR	
	attack		remittance		attack		remittance					
	M* No. %	F * No. %	M No. %	F No. %	M No. %	F No. %	M No. %	F No. %	M No. %	F No %	M No. %	F No. %
1-17 year	0 0%	1 9%	0 0%	0 0%	1 14.2%	0 0%	1 14.2%	2 14.3%	0 0%	0 0%	0 0%	4 40%
18-40 year	1 20%	6 54.6%	4 57.2%	3 25%	2 28.6%	3 42.8%	2 28.6%	8 57.2%	0 0%	0 0%	6 85.7%	5 50%
≥41 year	4 80%	4 36.4%	3 42.8%	9 75%	4 57.2%	4 57.2%	4 57.2%	4 28.5%	0 0%	0 0%	1 14.3%	1 10%
Total	5 31.3%	11 68.7%	7 36.8%	12 63.2%	7 50%	7 50%	7 33.3%	1 4 66.7%	0 0%	0 0%	7 41.2%	10 58.8%
	16 45.7%		19 54.3%		14 40%		21 60%		0 0%		17 100%	
	35 50%				35 50%				17			
	70											

After amplification of 16SrRNA gene target by polymerase chain reaction and electrophoresis by 2% agarose gel, bands of amplified gene of *Chlamydia pneumonia* were showed in Figure (3) for patients and (4) for control group.

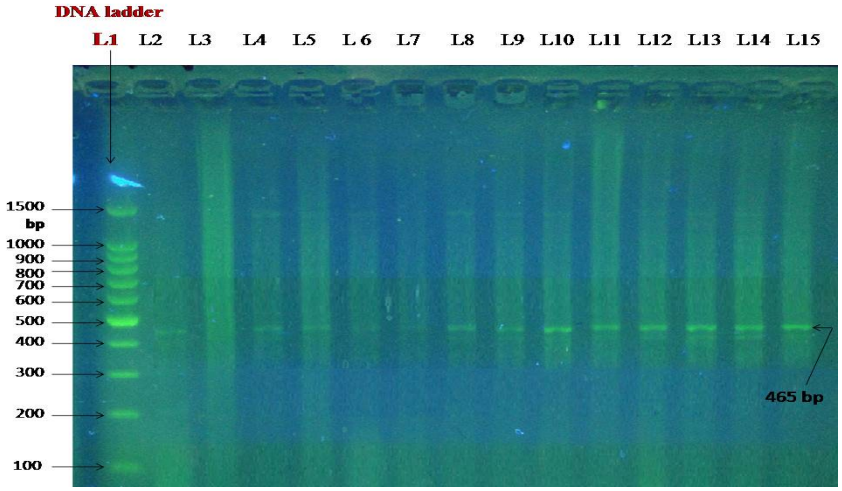

Fig. 3: The result of agarose gel electrophoresis (2%) with redsafe staining. Bands of amplified 16SrRNA gene of *Chlamydia pneumoniae* obtained from blood samples of patients. Note that (L2, L3, L4, L5, L6, L7, L8, L9, L10, L11, L12, L14, L15) were positive while (L3) were negative. DNA ladder with (100-1500 bp) on the left (L1) was used as DNA molecular weight marker.

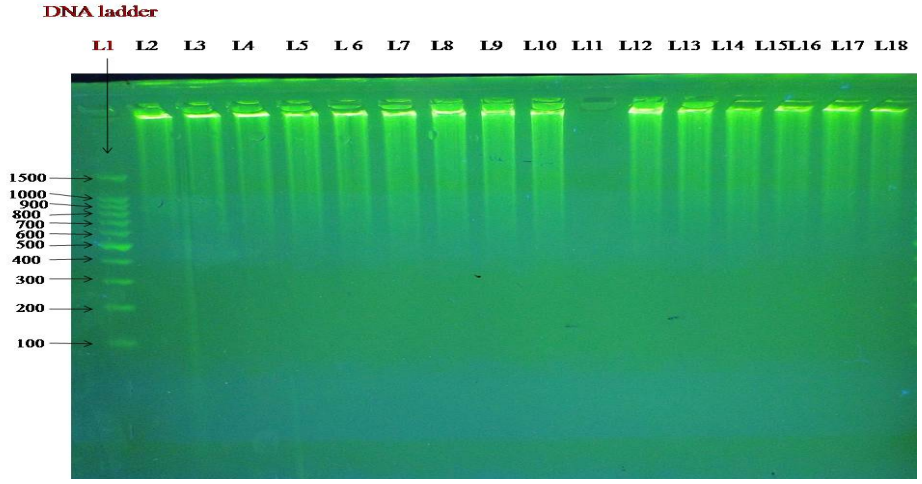

Fig. 4: The result of agarose gel electrophoresis (2%) with redsafe staining. Bands of amplified 16SrRNA gene of *Chlamydia pneumoniae* obtained from blood samples of control group. Note that all of samples were negative. DNA ladder with (100-1500 bp) on the left (L1), was used as DNA molecular weight marker.

Table 3: Age and sex distribution of PCR results of throat wash samples for patients and control group.

Age group	Patients group								Control group			
	+ve PCR				-ve PCR				+ve PCR		-ve PCR	
	attack		remittance		attack		remittance					
	M no. %	F no. %	M no. %	F no %	M no. %	F no. %	M no. %	F no. %	M no. %	F no. %	M no. %	F no. %
1 -17 year	0 0%	0 0%	0 0%	0 0%	1 12.5%	0 0%	1 9.1%	2 16.6%	0 0%	0 0%	0 0 %	4 40%
18 - 40 year	0 0%	2 100%	0 0%	0 0%	3 37.5%	7 50%	6 45.5%	4 33.4%	0 0%	0 0%	6 85.7%	5 50%
≥ 41 year	2 100%	0 0%	1 100%	0 0%	4 50%	7 50%	4 36.4%	6 50%	0 0%	0 0%	1 14.2%	1 10%
Total	2 50%	2 50%	1 100%	0 0%	8 36.4%	14 63.6%	11 47.8%	12 52.2%	0 0%	0 0%	7 41.2%	10 58.8%
	4 80%		1 20%		22 48.8%		23 51.2%		0 0%		17 100%	
	5 10%				45 90%							
	50								17			

B- Throat washes specimens:

Out of fifteen tested throat wash specimens, five (5) (10%) of them showed positive PCR for 16SrRNA of *Chlamydia pneumoinae*. Four (80%) of them were from adult patients at attack status and one (20%) of them in remittance stage. Throat wash specimens from adults showed more positive result from child and most of them were in attack status.

Regarding this category, non significant difference, ($P > 0.5$) was found between males and females, and all control group individuals showed negative PCR results, Table (3) and Fig.(5).

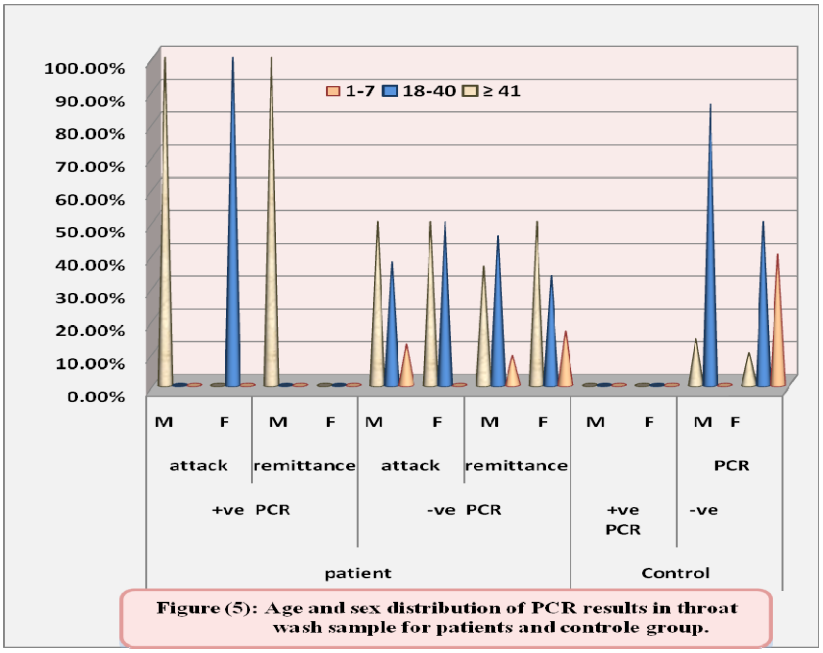

Figure (5): Age and sex distribution of PCR results in throat wash sample for patients and controle group.

After amplification of 16SrRNA gene target by polymerase chain reaction and electrophoresis by 2% agarose gel, bands of amplified gene of *Chlamydia pneumonia* were showed in Figure (6) for patients and (7) for patient and control group.

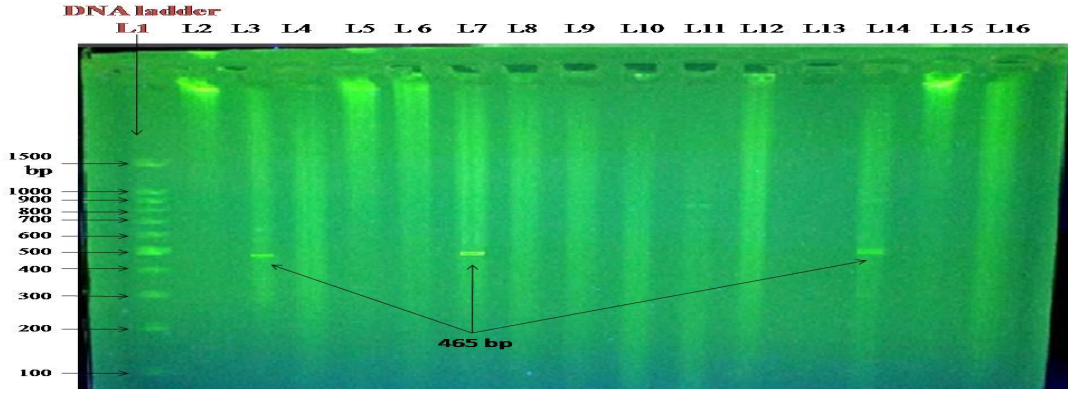

Fig. 6: The result of agarose gel electrophoresis (2%) with redsafe staining. Bands of amplified 16SrRNA gene of *Chlamydia pneumoniae* obtained from throat wash samples of patients. Note that (L3, L10, L14) were positive, while (L1, L2, L5, L6, L7, L8, L9, L11, L12, L13, L15) were negative. DNA ladder with (100-1500 bp) on the right (L16) was used as DNA molecular weight marker.

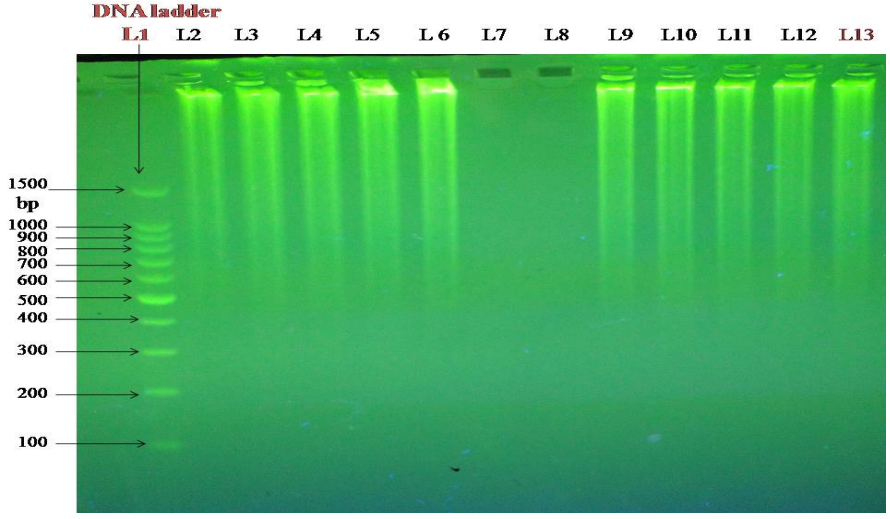

Fig. 7: The result of agarose gel electrophoresis (2%) with redsafe staining. Bands of amplified 16SrRNA gene of *Chlamydia pneumoniae* obtained from throat wash samples of patients (L2 to L6) and control group (L9 to L13). Note that all samples were negative. DNA ladder with (100-1500 bp) on the left (L1) was used as DNA molecular weight marker.

3- IgE Specific for *Chlamydia pneumoinae*:

Out of (54) tested sera from asthmatic patients, 37 of them showed positive ELISA test for IgE specific for *Chlamydia pneumoniae*, (17 patients at attack and 20 of them were at remittance). Adult females (24) (64.8%) showed higher IgE positive results than males (P=0.003). All tested serum samples (15) from control group individuals showed negative IgE specific for *Chlamydia pneumoniae* (Table-4). No significant difference was found between attack and remittance asthmatic patients who showed positive ELISA for specific IgE for *Chlamydia pneumoinae*, (P=0.62). Positive correlation was found between the results of IgE specific for *Chlamydia pneumoniae* and PCR results for blood and throat wash specimens in both attack and remittance patients (P=0.00), Table (4) and Figure (8).

Table 4: Age and sex distribution of specific IgE for *Chlamydia pneumoniae* in sera of patients and control group.

Age group	positive				negative				Control			
	Attack		Remittance		Attack		Remittance		positive		negative	
	M * No. %	F * No. %	M No. %	F No. %	M No. %	F No. %	M No. %	F No %	M No. %	F No. %	M No. %	F No. %
1 – 17 year	0 0%	1 7.7%	0 0%	0 0%	0 0%	0 0%	1 25%	1 12.5%	0 0%	0 0%	1 12.5%	2 28.6%
18 – 40 year	1 25%	7 53.9%	4 50%	4 33.3 %	1 25%	0 0%	2 50%	4 50%	0 0%	0 0%	6 75%	3 42.8%
≥ 41 year	3 75%	5 38.4%	4 50%	8 66.7 %	3 75%	1 100%	1 25%	3 37.5%	0 0%	0 0%	1 12.5%	2 28.6%
Total	4 23.5%	13 76.5%	8 40%	12 60%	4 80%	1 20%	4 33.3%	8 66.7%	0 0%	0 0%	8 53.3%	7 46.7%
	17 45.9%		20 54.1%		5 29.4%		12 70.6%		0 0%		15 100%	
	37 68.5%				17 31.5%				15			
	54											

M*: male
F *: female

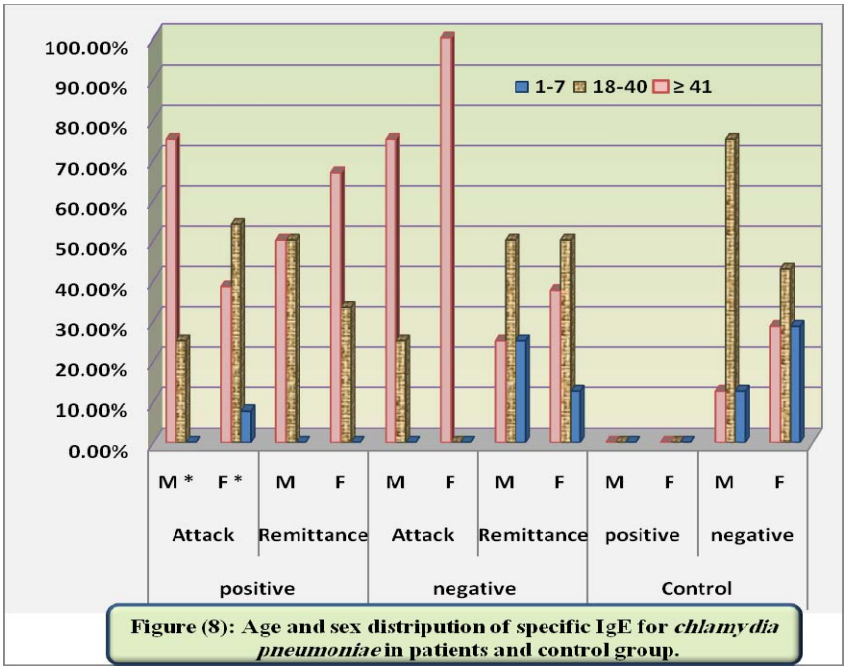

Figure (8): Age and sex distripution of specific IgE for *chlamydia pneumoniae* in patients and control group.

DISCUSSION

Infective asthma (Asthma imposed by infective agent mediators and allergens) had been well recognized by many pioneers who found that asthma could be induced by one or more of different bacterial agents, viruses, fungi and even parasites Oiling, BRADA 1996, Cunningham 1998, kraft, 2000, Fernandez 2001, Lafi 2004, lafi 2008, Mnadero and Lprise 2010).

We found increased rate of asthma in female adults (55.8%) in contrast to males ratio (44.2%), this was in accordance with that reported by (Lafi 2004, 21 Zhao *et al*. 2001, Shames *et al*. 1999). This difference among sexes might be due to, environmental, domestic and occupation effect on females (makes them more exposed to infective and non infective environmental allergens than males as well as the effect of sex

hormones that modulates immune status in females (Zhao *et al*. 2001). Decreased number of asthmatic children in this study was attributed to the method of sampling, patients included in this study were attending Ramadi General Teaching Hospital and the Clinics at this hospital were dealing with adult patients. So in order to include more asthmatic children data, specimens should be collected from patients were attending child hospital.

Regarding PCR test results, adult females showed more positive PCR results for 16S rRNA of *Chlamydia pneumoinae* in their blood specimens, and negative results in blood from control individuals, this indicates presence of *Chlamydia pneumoinae* in asthmatic patients. This was found also by (Harju *et al*., 2006; Mitchell *et al*., 2009, Zahang 2000) positive titers of IgE specific for *Chlamydia pneumoinae* antigen in adult asthmatic patients particularly females indicated the role of *Chlamydia pneumoniae* allergens in of Atopy and astma induction. This was in acceptance with that mentioned by (Brada 1996 Kraft 2000, Numazakei 1990, Krull *et al*. 2006, Kokabas 2008) who showed that microorganisms were imposed in infective asthma through their allergens.

Decreased rate of positive results of PCR test in throat wash was ought to the fact that Chlamydia pneumoniae is an obligate intracellular pathogen (Brook, and Caroll, 2007; Krull *et al*. 2006) and it need to bind host cells and free cells wash out with saliva, so it is difficult to see free chlamydial cells in saliva and throat wash specimens (Harvy, 2008). Our findings in PCR test for throat wash was nearly in accordance with finding of Harjull *et al*. 2006 and disagree with that of Zhang *et al*. 2000, Kokabas *et al*. 2008).

Positive correlation between PCR test results and IgE specific for *Chlamydia pneumoniae* confirm our findings through the importance of entrance of the pathogen to the host cell and immune activation (Male *et al*. 2006, Krull 2006, Mora *et al*. 2009).

We can conclude that *Chlamydia pneumoniae* involved in upper respiratory tract infection through the presence in host tissue and immune induction through Th2 activation and release of IgE specific for its owned antigens and later on induction of type one hypersensitivity (Anaphylaxis) as indicated by the positive titers of IgE specific for *Chlamydia pneumoniae*. We recommend screening tests for microbes (*Chlamydia pneumoniae*) in asthmatic patients and suiTable anti *Chlamydia pnemoniae* treatment should be used for asthmatic patients in addition to that we recommend further studies on other microorganisms role in asthma in both adults and children to relief infective asthma properly.

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ARABIC SUMMARY

تشخيص الحامض النووي الريبى عيار 16S للكلاميديا الرئوية وإلضد IgE الخاص بها في مرضى الربو

حاتم عبد الرزاق السبهانى[١] و شهاب احمد لافى[٢] ومشتاق طالب صالح العقيلى[٣]

١- وزارة الصحة جمهورية العراق دائرة صحة الانبار .المستشفى التعليمي الرمادي

٢- قسم الأحياء المجهرية كلية الطب جامعة الانبار غرب العراق.

٣- كلية الصيدلة جامعة الانبار غرب العراق.

Shehab58@yahoo.com

توطئة:

الكلاميديا الرئوية هي واحدة من الجراثيم المجبرة داخل الخلية والتي تسبب التهاب المجارى التنفسية العليا. الجراثيم متهمة بكونها احد مسببات الربو القصبي بميكانيكية مختلفة ومن هذه الجراثيم هي الكلاميديا الرئوية.

أهداف الدراسة:

هدفت هذه الدراسة لبيان نسبة إصابة مرضى الربو بجرثومة الكلاميديا الرئوية من خلال تحديد الحمض الريبى عيار 16S الخاص بها باستخدام تفاعل سلسلة البلمرة الجينية PCR وكذلك بيان دور هذه الجرثومة بإحداث الربو من خلال الكشف عن الضد IgE الخاص بها .

طرائق العمل :

أجريت هذه الدراسة على (١٢٠) مريضا ولأعمار تراوحت من (٢-٨٤) عاما راجعوا العيادة الاستشارية للربو والحساسية في مستشفى الرمادي العام للفترة من كانون ثاني ٢٠١١ ولغاية آذار ٢٠١١ واختير ثلاثين شخصا آخرين عشوائيا من كلى الجنسين سالمين من كل إصابة ليمثلوا عينة ضابطة للدراسة.

أخذت عينة دم (٢مل) وغسول الحنجرة لكل مريض واستخدمت لعزل الحمض النووي الريبى عيار 16S لجرثومة الكلاميديا الرئوية باستخدام تفاعل سلسلة البلمرة الجينية PCR كما واستخدمت ٧٠ عينة مصل دم من المرضى للكشف عن الضد IgE الخاص بجرثومة الكلاميديا الرئوية باستخدام كشف الاليزا.

النتائج :

تبين من اختبار ٧٠ عينة من المرضى إن (٣٥) (٥٠%) منهم أعطت نتيجة موجبة لتفاعل سلسلة البلمرة الجينية PCR كما وكان اغلبهم من الإناث البالغات (٢٢) عينة عشر (١٠) منها من مريضات في حالة نوبة الربو، بينما جميع عينات الأشخاص من المجموعة الضابطة أعطت نتيجة سالبة لهذا الاختبار . كانت نتيجة (٣٧) عينة من أصل (٥٤) عينة مصل دم موجبة لاختبار الضد IgE الخاص بجرثومة الكلاميديا الرئوية وكان اغلبها أيضا من الإناث البالغات (٢٤) (٦٤.٨%) وعليه لوحظ فرق معنوي بين نتائج الذكور البالغين والإناث البالغات (P<.0.05)

جميع العينات المفحوصة من المجموعة الضابطة (١٥) أعطت نتيجة سالبة لاختبار IgE . لوحظ أيضا ترابط بين نتائج هذا الاختبار ونتائج وتفاعل سلسلة البلمرة الجينية PCR في مرضى الربو ممن هم في نوبة الربو والراحة.

الاستنتاجات:

تبين من الدراسة أن الكلاميديا الرئوية لها دور في الربو القصبي من خلال إصابتها للالتهاب المجارى التنفسية السفلى وإحداثها الربو من خلال حث تكوين محدثات التحسس ومنها الضد IgE وفى كلى الجنسين .