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Molecular Diagnosis of *Chlamydia pneumoniae* in Coronary Heart disease Patients in Diyala City

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Abstract: *Chlamydia pneumoniae* has been highly associated with & implicated in the procoagulatory & inflammatory component of atherosclerosis. The present study has been designed to detect *C. pneumoniae* DNA and *C. pneumoniae* IgG and IgM antibodies among Iraqi patients with coronary heart disease and the association between the bacterium and risk factors. The study involved 71 patients diagnosed with (angina, myocardial infarction and atherosclerosis) and 30 healthy controls. Out from 71 patients, 28.2% were smoking, 26.8% were diabetic and 45.1% were hypertensive. Detection of anti- *C. pneumoniae* IgM and IgG antibodies was done by Enzyme-Linked Immunosorbent assay. *C. pneumoniae* IgG antibodies were detected in 57.7% of patients and 14.6% of healthy controls, while seropositivity of *C.pneumoniae* IgM antibodies were detected in 9.9% patients. DNA were extracted from blood samples and PCR was used for detection of the *C. pneumoniae* genes. Thirty DNA samples of the coronary heart disease patients were amplified by PCR using three genes *16SrRNA* with 194bp and outer membrane protein (*OMP*) and were included as internal with 239bp and outer with 499bp both specific gene for *C. pneumoniae*. The results of all genes revealed that 10 patients (33.3%) were positive , while it was negative for all control group.

Key Words: *C. pneumoniae*, CHD, Molecular detection, PCR.

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Introduction

Chlamydia pneumonia is a Gram-negative, obligate intracellular bacterium coccoid or rod-shaped, non-motile, that causes a plethora of illnesses in humans (1). CHD, a subset of cardiovascular disease (CVD) that accounts for more than 50% of all CVD events in adults under the age of 75 (2). CAD is the number one killer in the developed world, with over 7.4 million deaths attributed to CAD in 2012 (3). There are many globally considerable diseases that have been linked with chronic *C. pneumoniae* infections and one of them is atherosclerosis (4). Atherosclerosis, an important pathological characteristic in CHD, is closely correlated with inflammation (5). An enzyme-linked immunosorbent assay (ELISA) was developed as an easier technique to perform the detection of antibody titers against Chlamydia in patient biological fluids (6). Hypertension, obesity,

hypercholesterolemia, smoking and diabetes mellitus can partly determine the pathogenesis of the disease , although they are considered as the conventional risk factors for atherosclerosis (8). Polymerase chain reaction (PCR), in-situ hybridization method & enzyme immunoassay protocols are diagnostic methods used to detect *C. pneumoniae* in human samples. Chlamydial genome parts, particularly genes encoding 16S rRNA & major outer membrane protein (OmpA) are usually targeted by (PCR-based approach (9). PCR technique is a convenient, important & potential diagnostic tool to detect *C. pneumonia* reliably and rapidly due to its ability to amplify small amounts of specific nucleic acid. Detection of *C. pneumonia* DNA is described using many different targets (16S rDNA, pmp4, MOMP) primers and reaction protocols (10,11). Polymerase chain reaction-based detection of *C. pneumoniae*-unique DNA sequences: this approach is both specific and sensitive, it can also be quantitative. PCR will allow infection burden to be correlated with clinical phenotypes (12-14). The aim of the present study was to investigate the presence of *C. pneumoniae* DNA among Iraqi patients with atherosclerosis and the association between the bacterium and atherosclerosis risk factors.

Materials and methods

The study included 71 patients with (angina, myocardial infarction and atherosclerosis) who were admitted to the cardiology clinics of Baqubah Hospital Diyala, Iraq between August 2016 to November 2016.The CHD patients included 37 males and 34 females. Age and sex-matched healthy control who have been collected form blood bank donors after pre-examination and scanning from all diseases, were also included. Apparently healthy control subjects comprised 18 males and 12 females. Written informed consent has been taken from all individuals who participated in the study. This study has been approved by the ethics committee standard of Baqubah Hospital.

II-Samples collection

Five ml of blood samples were collected from study patients and control groups, 2ml in an EDTA tube and 3ml in gel-containing tubes (Primum, Austria). Serum samples were obtained by centrifuging blood at 3,000 rpm and stored at -20°C until analyzed. Blood in the EDTA vials were used for DNA extraction by G- spin DNA extraction kit.

Molecular detection

I-DNA extraction from whole blood

DNA was isolated from the whole blood specimen which was obtained from all study groups using G- spin DNA extraction kit (iNTRON Biotechnology, Gyeonggi-Do,Korea) pursuant to the guidelines of the manufacturer. DNA was extracted and stored at -20°C until tested.

II-Polymerase chain reaction

Three primers were used to amplify *C.pneumoniae* DNA. *16SrRNA*, Outer membrane protein (*OMP*) internal and outer gene, both specific for detection *C.pneumoniae* with Amplicon sizes: 194bp, 249bp, 499bp respectively, as shown in (Table 1, 2, 3).

Table (1): The specific primer of gene 16SrRNA.

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- GTATGAAACTCTTGATCGTCT- 3'	49.6	38.1	194bp
Reverse	5'- CCGCATGATCAGGTTAGTAAT- 3’	53.3	42.9	

Table (2): The specific primer of the outer membrane protein (*omp*): Internal

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- TGTCCAAGCGGTGAAACAAG- 3'	55.9	50	239bp
Reverse	5'- CAACCGTGACCCATTTACTG- 3’	53.7	50	

Table (3): The specific primer of outer membrane protein (*omp*): Outer

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- ATGATCGCGGTTTCTGTTGCCA- 3'	59.7	50	499bp
Reverse	5'- GAGCGACGTTTTGTTGCATCTC- 3’	57	50	

By using Maxime PCR PreMix kit (i-Taq) for diagnosing genes, each tube contains 5µl PCR PreMix, 1µl for each primer (Forward, Reverse),1.5µl of DNA samples were added and 16.5µl of DNase/RNase-free distilled water. By using thermocycler PCR, optimization was done for each gene. PCR programs was done according to each gene as following, listed in (Table4).

Table (4): PCR program for amplification of *C.pneumoniae 16S rRNA* gene and *OMP* (outer and internal) gene.

Phase	Tm (°C)	Time	No. of cycle
Initial Denaturation	95°C	3 min.	40 cycle
Denaturation	95°C	30sec	
Annealing	56°C* 60°C** 54°C***	30sec	
Extension	72°C	45sec	
Final Extension	72°C	10 min.	

***Annealing TM:* * 16S *rRNA* gene, ** outer gene , *** internal gene.**

Gel Electrophoresis

The amplicons were determined by 2% agarose gel electrophoresis,stained with red dye at 5 volt/cm² for 90mins and visualized by gel documentation system**.**

Statistical analysis

Statistical data analysis were used to analyze and assess the study results by applying the version (14) of the statistical package (SPSS) including: Statistical tables, frequencies, and percentages, with mean value, and

standard deviation, and standard error of mean values. Odds Ratio of the related rates including: Measurement of the association strength between the presence of a factor and the occurrence of an event, (95%) Confidence interval for population Odds ratio values.

Results

A total of 71 patients 34(47.9%) females & 37(52.1%) males with a mean age (62± 11.5) years (range of 40-85 years) have participated in this study. Major risk factors hypertension, diabetes mellitus and smoking were selected as risk factors among CHD patients and controls as shown in Table(5). Out of 71 patients, 28.2% were smoking, 26.8% were diabetic with highly significantly difference (P<0.01). While the percentage of hypertensive among patients was found to be 45.1% with non significant difference (P>0.05).

Table (5): Percentage of the risk factors among CHD patients

Risk factors	No.	%
Smoking	20	28.2
Hypertension	32	45.1
Diabetes mellitus	19	26.8

Detection of *C.pneumoniae* IgG and IgM antibodies by ELISA.

C. pneumoniae IgG antibodies were detected in 57.7% patients and 14.6% controls with highly significant difference at P<0.01. While *C. pneumoniae* IgM was only found in 7(9.9%) of patients with non significant difference P>0.05, as listed in table (6).

Table (6): Prevalence of *C.pneumoniae* IgG, IgM antibodies among studied samples.

Anti *C.pneumoniae* antibodies		Patients No.%	Controls No.%	C.S. (*) P-value
IgM	Pos.	7 (9.9%)	0 (0%)	P = 0.184
	Neg.	64 (90.1%)	30 (0%)	NS
IgG	Pos.	41 (57.7%)	7 (14.6%)	P=0.002
	Neg.	30 (42.3%)	23(43%)	HS

HS: Highly Sig. at P<0.01; NS: Non Sig. at P>0.05; [Testing based on Binomial Test].

Molecular diagnosis of *C.pneumoniae* DNA

Detection of *C.pneumoniae* DNA in whole blood samples as a prospect marker of CHD was tested. Polymerase chain reaction for detection of *C. pneumoniae* was used for 30 cases and 10 control group. Amplification of *16SrRNA*(149bp), internal (239bp) and outer (499bp) *omp* genes for *C.pneumoniae* was done. The results of

all genes revealed that 10 patients (33.3%) were positive ,while it was negative for all control group.

The 16SrRNA gene was amplified by forward and reverse primers. Our result showed positive PCR products for *16SrRNA* gene with molecular size 194bp in Gel electrophoresis compared with the ladder (figure 1).

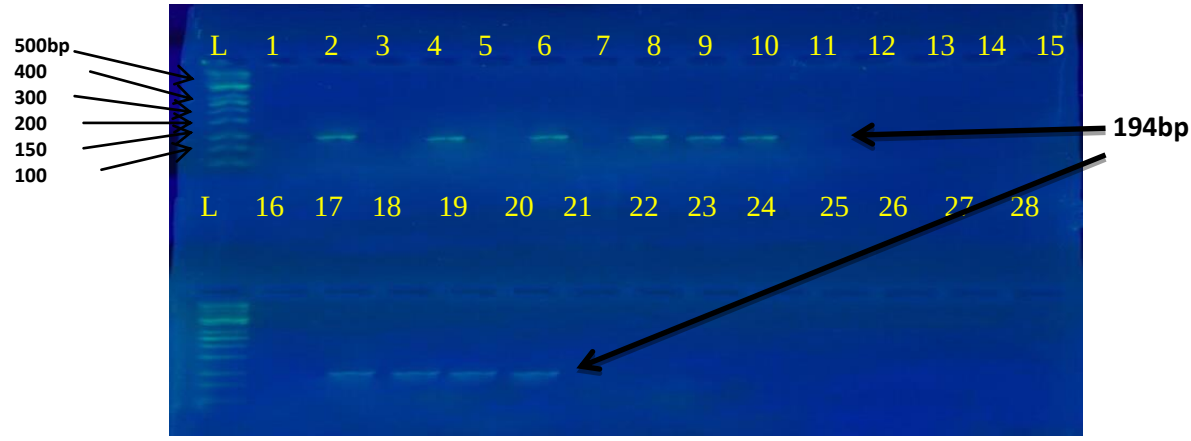

Figure (1): Products of 16SrRNA gene was Gel electrophoresis in 2% agarose gel at 5 volt/cm^2 for 1:30 hours, Lane: Ladder DNA (100-10000). Lane 2,4,6,8-10,17-20: band of C.pneumoniae DNA. Lane 1,3,5,7,11-16: no bands.

The internal *OMP* gene was amplified by forward and reverse primers. Our result showed positive PCR products for *internal* gene with molecular size 239bp in Gel electrophoresis compared with the ladder(figure 2).

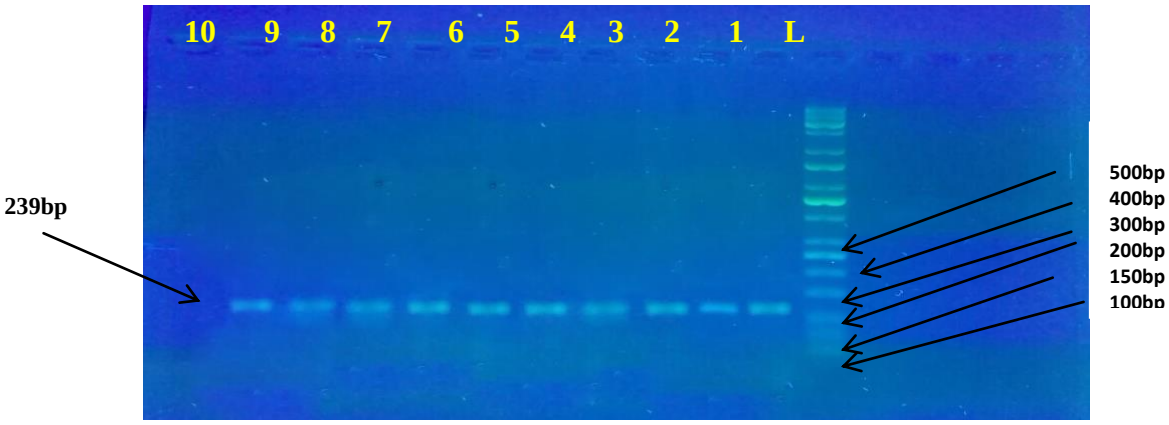

Figure (2): Gel electrophoresis of omp gene (internal), 2% agarose gel at 5 volt/cm^2 for 90 mins, Lane: Ladder DNA (100-10000). Lane1-10: band of C.pneumoniae DNA.

The outer *OMP* gene was amplified by forward and reverse primers. Our result showed positive PCR products for *internal* gene with molecular size 499bp in Gel electrophoresis compared with the ladder, as mentioned in (figure 3).

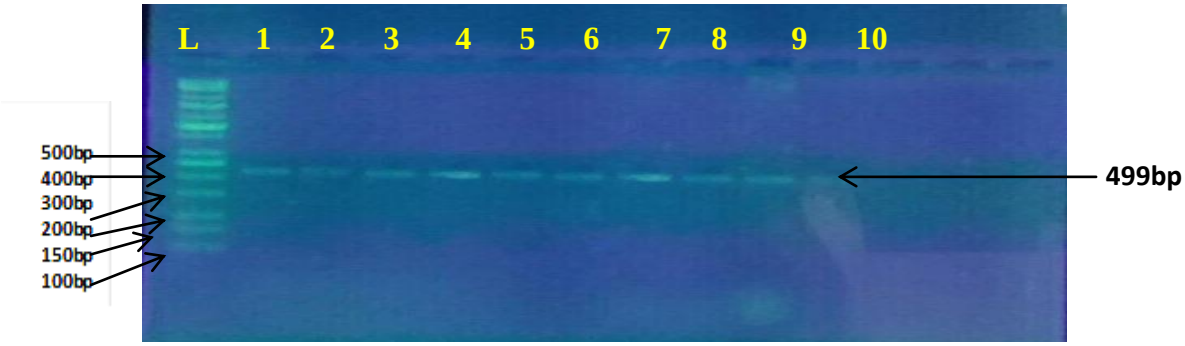

Figure (3) : Gel electrophoresis of *OMP* gene(outer),2% agarose gel at 5 volt/cm^2 for 90mins, Lane: Ladder DNA (100-10000).1-10: band of C.pneumoniae DNA.

Discussion

CHD is a complex, multifactorial disease. Several studies have reported a possible association between infection with atherogenesis and microbial agents such as *Chlamydia pneumoniae*. Human Cytomegalovirus, Herpes Simplex Virus 1, and Epstein Barr Virus have been widely investigated for their possible role in atherosclerosis development (15). Many risk factors are correlated with such grave conditions like obesity, hypertension, smoking, hypercholesterolemia, diabetes & infections. Infection was considered in the studies as a potential risk factor for such health problems. Our finding is consistent with the result of Zaki *et al* who indicated that hypertension is the predominant risk factor that is found among patients (82.5%) followed by obesity (68.7%) and DM (33.8%) (16). Another study presented different risk factors like hypertension and dyslipidemia which showed a significant difference compared to healthy control (15). While, Esmat, 2015 found the major risk factors was DM as (59.5%) for the patient and (28.57%) of controls, while Smoking patients and control were (58.1%) and (28.57%) respectively (11). In addition, The results of the current study disagreed with the studies of Assar *et al*. and Dabiri *et al*. who found a high prevalence of *C.pneumoniae* infection among smokers in comparison to non-smokers (12)(13). Our findings are consistent with the results of Ali *et al.* who found the percentage of seropositive patients with diabetes, smoker to CHD (14). These findings assume that susceptibility to *C. pneumoniae* infection is elevated by smoking (16). The majority of proatherogenic actions of smoking, like induction of endothelial dysfunction, interference with blood coagulation & promotion of lipid peroxidation reverse themselves shortly after smoking cessation. Such patho-mechanisms can be related to the development of vascular diseases among smokers due to the facilitating effects of smoking on the manifestation of different persistent infectious illness types. (20). One-third (33.3%) of the studied group gave a positive result to *C. pneumoniae* DNA in the whole blood, suggesting that *C. pneumoniae* considered one of a risk factor for CHD. Patients groups were carrying bacteria truly because of the fact that PCR assay is observed as a golden diagnostic method (22). Studies on *C. pneumoniae* are limited in Iraq,

Al-Masoudi *et al*. found that (7.7%) were positive in their blood samples for target specific of *C. pneumoniae* gene *16SrRNA* with molecular length 460 base pairs, while found that (80%) were positive to *C. pneumoniae* omp gene with molecular length 207 bp (23). Bacterial phylogeny and taxonomy is done by use of *16SrRNA* gene sequences , as it is the most communal housekeeping genetic marker used and the 16SrRNA presence in virtually all bacteria, often existing as a multi-gene family or operons ith (1,500 bp) large enough for informatics purposes (24). In the detection of *C.pneumoniae* gene , Esmat *et al*., 2015 showed that all controls were negative , while 28 patients (37.84%) were positive, with p-value 0.05.(16). The Iranian study performed by Assar *et al*. 2016 disclose *C. pneumoniae* DNA in 30% cases in atheroma plaques versus 6% in controls are standardized(17). This was approved by others (18)(25). While others like Sadeghian *et al*. found that all the control samples were negative , while Positive PCR result for *C. pneumonia* was seen in 1(3.3%) sample among the 30 coronary artery tissues with atherosclerosis, that indicate a weak relationship between *C. pneumoniae* and atherosclerosis(26). We used PCR for detection of C. pneumonia; this test appears to be more sensitive than cell culture (27). In addition, Chatzidimitriou showed that only 15(12.3%) atherosclerotic sampless of patients were positive for *C. pneumoniae* DNA by PCR detection(23), because of the presence of low density microorganisms in the atherosclerotic lesion or because of the high specificity of PCR technique. Al-Younes *et al* reported *C. pneumoniae* DNA in only 7.8% of patients with CHD & in 9.2% of the control group (24). This study clearly found that both PCR using whole blood specimens & serology are efficient in the diagnosis of *C. pneumoniae* carriers & , accordingly, it is a reliable tool to link CAD with this pathogen (25). A highly significant sero-prevalence of *Chlamydia pneumoniae* IgG (57.7%) was found in our study in atherosclerosis patients compared with the control group (14.6%). The result of this study is consistent with Khudair *et al*. who reported a high positive rate of anti- *C. pneumoniae* IgM & IgG antibodies in patients group compared to the control group , & the positivity of anti-*C.pneumonia* IgM & IgG antibodies in patients group was 6.42% & 50.7% respectively, while in the control group it was 0% for the two types (26). In addition, the current results in this study were in agreement with other studies like a study by Swetha *et al*. who studied *Chlamydia pneumoniae* IgG as well as the study of the association of chronic *C. pneumoniae* infection with coronary artery disease(27). Another study by Agarwal *et al*. , where the relationship of chronic *C. pneumoniae* infection with CAD was investigated & found that the percentage of IgG antibodies was (61%) in CAD group in comparison with (38%) in the control group(28). Many research studies using several techniques tried to make a link between *C. pneumoniae* chronic diseases such as atherosclerosis. IgG antibody possession by *Chlamydia pneumoniae* demonstrates either a previous exposure (with antibody persistence) or a chronic or latent infection (29). However, two different tests for detecting anti-*C. pneumoniae* antibodies were used. Infections are linked to atherosclerosis by increasing

body evidence. Therefore, it is proposed that other risk factors could be interacted with infections for vascular disease, enhancing the endothelial damage & production of atherosclerotic plaques (30). Additionally, our results are concordant with Al-Duliami who found a high prevalence of anti-*C.pneumoniae* IgG antibodies detected among CHD patients (66.67%) when compared with the control group(31). In an Indian study, Swetha *et al*. showed a highly positive anti- *C. pneumoniae* IgG antibodies associated with CHD risk (27). ELISA is usually used for the diagnosis of *C.pneumoniae* infection because the results can be interpreted objectively, and because it is a convenient rapid method (32). Molecular-analysis-based assays, such as PCR, have recently been developed for the rapid and sensitive detection of *C. pneumoniae* (33). Therefore, it is postulated that other risk factors for vascular disease, enhancing the endothelial damage & production of atherosclerotic plaques could interact with infection (34). Confirmation of serological assays such as PCRs with tests that demonstrate the existence of the organism is recommended, not with standing their limitation. Application of PCR as a reference test allows a more accurate calculation of the analytical sensitivity for the detection of active (acute or chronic) *C. pneumoniae* infection. Although many in-house PCR assays have shown a high specificity & sensitivity, most MIF tests & ELISAs depend on the detection of antibodies against the whole chlamydial elementary bodies (EBs), which explains their inherent shortcomings in regard to cross-reactivities between Chlamydia species & other, even unrelated, microorganisms (35).

Conclusion

It can be concluded from this study that results support the hypothesis of association of *C. pneumoniae* infection with atherosclerosis and confirming that *C. pneumoniae* is one of the risk factors of Coronary Heart Disease. Hypertension indicates one of the risk factors for the disease.

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