Prevalence of Chlamydia pneumoniae by Real time PCR in referred patients with respiratory syndrome to clinic center of infectious diseases

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Abstract: Introduction: Chlamydia pneumonia is the one of the most common reasons of community acquired pneumonia that can disturb cholesterol homeostasis in human and leading causes of coronary artery diseases and death. The purpose of the current study were to determine prevalence of Chlamydia pneumoniae by Real time PCR in referred patients with respiratory syndrome to infectious diseases clinic of university hospital. **Methods and Materials:** This study was an analysis of epidemiological descriptive study and Blood samples were collected for Chlamydia pneumoniae was tested by Real time PCR. The desired data using descriptive statistics such as frequency distribution tables of agreement, mean, standard deviation and were compared and analyzed with spss18. **Results:** In this study, 104 patients were examined, 43 were men and 24 are women. and, 100% of the samples were negative for Chlamydia pneumoniae. **Discussion:** In this study all of the samples were negative for Chlamydia pneumoniae; numerous studies have been conducted in other parts of the world as well as a small percentage of Chlamydia pneumoniae from blood samples were positive. The reasons are low number of samples, low levels of bacteremia, the intracellular organisms, Excessive antibiotics prescription without any indications.
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Introduction

Chlamydia pneumoniae is a rod-shaped Gram-negative bacterium of the Chlamydiaceae family that may be up to 1µm in length. Chlamydia pneumoniae exists as an elementary body when outside of a host. In this conformation, the bacterium is not biologically active, but is able to withstand environmental stresses until in reaches a new host where, upon phagocytosis by the host lung cell, it transforms to areticulate body which is then able to replicate. After replication, the reticulate body reverts back to an elementary body, is released from the infected cell by lysis and is then able to infect other cells. The infection and lysis of host lung cells causes pneumonia which results in symptoms including cough, fever and nasal congestion that can lead to sinusitis, pharyngitis and laryngitis.

Chlamydia pneumoniae is a pathogenic bacterium that causes pneumonia and respiratory infections in humans and also one of the important etiology of Atypical pneumonia (1).Chlamydia pneumoniae can engender Bacterial pneumonia on teenagers and Adolescents individuals (2). Prevalence of Chlamydia pneumoniae in Community Acquired pneumonia is 6 to 20 percent (3, 4) and there is a significant Relationship between Chlamydia pneumoniae and coronary vessels (5). There is several kinds of Diagnostic assays For Chlamydia pneumoniae as: Serology with ELISA, IFA , PCR and Culture (6, 7).

Culture of Chlamydia is very difficult became of needs to eukaryotic cells and it is a non sensitive assay (8). Therefore one of the best assays' for Diagnose of Chlamydia pneumoniae is PCR; Because that is available and high specific (9-12),Hence we carried out this study for Discover Chlamydia pneumoniae in patients with Atypical pneumonia.

Material and methods

This study after Sanction in ethical committee of lorestan university of Medical Science has been carried out in spring of 2013; On 98 patients with Atypical Respiratory pneumonia Symptoms and signs that Referred to Infections Diseases clinic center in shohada Ashaier university hospital. This patient has had Dry cough, Dyspnea with or without fever That after clinical Examination via Infections Diseases Specialist, patients have Referred to hospital library and has been tacked 5cc blood from them hence after samples centrifugation , have been sent to referral lab

for Real time PCR. We used Quantification of Chlamydia pneumoniae kits from primer Design CO.

Diagnose and treatment of Chlamydia is very important because of role in CAD and lipoprofile balance and also probable role in create or deteriorated unknown etiology Syndrome or so multiple sclerosis, guillain-barre syndrome and Molecular – cellular disturbances. Main protein gene was (omp A) that in termed cycler has been propagated, growth and Increased then Compared with Negative and positive chart diagram control.

Principles of the Real-time PCR Test

A C.pneumoniae specific primer and probe mix is provided and this can be detected through the FAM channel. The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR.amplification, forward and reverse primers hybridize to the C.pneumoniae DNA/cDNA. A fluorogenic probe is 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.

Positive control

For copy number determination and as a positive control for the PCR set up, the kit contains a positive control template. This can be used to generate a standard curve of C.pneumoniae copy number / CT value. Alternatively the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target C.pneumoniae gene worked properly in that particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a Post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To confirm the absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNAse/DNAse free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources of contamination should first be explored and removed.

Bench side Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

1. Pulse-spin each tube in a centrifuge before opening. This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. Reconstitute the kit components in the RNase/DNase-free water supplied, according to the table below. To ensure complete resuspension, vortex each tube thoroughly.

Real-time PCR detection

1. Prepare a reaction mix according to the tables below:

Include sufficient reactions for the standard curve wells 6 sample the negative control.

Table1. C.pneumoniae detection mix

Component	Volume
oasig™ 2 x qPCR MasterMix	10 µl
C.pneumoniae Primer/Probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final Volume	15 µl

2. Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.

3. Prepare sample DNA templates for each of your samples (suggested concentration 5ng/µl) in RNAse/DNAse free water. If the concentration of DNA is not known, then dilute your DNA sample reactions 1:20 (10µl of sample DNA and 190µl of water).

4. Pipette 5µl of diluted DNA template into each well, according to your experimental plate set up.

For negative control wells use 5µl of RNAse/DNAse free water. The final volume in each well is 20µl.

5. Preparation of standard curve dilution series.

1) Pipette 900µl of RNAse/DNAse free water into 5 tubes and label 2-6

2) Pipette 100µl of Positive Control Template (RED) into tube 2

3) Vortex thoroughly

4) Change pipette tip and pipette 100µl from tube 2 into tube 3

5) Vortex thoroughly

Table2. Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

6. Pipette 5µl of standard template into each well, according to your experimental plate set up. The final volume in each well is 20µl.

Table3. Amplification conditions using oasigTM 2x qPCR MasterMix

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37 °C
	Enzyme activation (if required)	15 mins	95 °C
50 Cycles	Denaturation	10s	95 °C
	DATA COLLECTION *	60s	60 °C

* Fluorogenic data for the control DNA should be collected during this step through the FAM and VIC channels
** Required if your Mastermix includes UNG to prevent PCR carryover contamination

Statistical Analyses

For collecting Demographic data (age, sex) we have used from questionnaire as: smoking, opium addiction, corticosteroid recipient, chronic respiratory syndromes. Fore Report of prevalence of Chlamydia pneumoniae we used from percentage and frequency But for compare frequency of Chlamydia according to underlying Diseases , sex ,age, smoking , opium addiction , corticosteroid consumption was applied from chi-square and fisher test. In the all study stages was gratuitous voluntary for patients exit and all of charges test was free for them. At last all data has been analyzed with spss 18 software package.

Results

Present Study carry out on 104 patients that Referred with Acute Respiratory Symptoms that has been evaluated mean of patients age was 35/2 ± 16/3 with minimum years and Maximum 89 years old. 49 patients (47/1%) were male and 55 (52/9%) were female (Table 4).

Twenty patients has had smoking history and 40 patients (38/5%) has had corticosteroid consumption. Opium addiction history was positive for 6 (5/8%) of patients. 2 (1/9%) of patient has had non-respiratory underlying disease so Diabetes Mellitus. But Asthma was (1/9%) bronchitis (1%), Asthma and bronchitis. Concomitant Disease was 1/9%, all of patient that have been evaluated with PCR assay; were negative for Chlamydia pneumoniae. Perhaps this study has been perform in a tertiary hospital, while the most of patients have been Received Antibiotic before Referred to our Infection center.

Discussion

All of samples in our study were negative for Chlamydia pneumoniae with PCR assay notwithstanding all of our patients has had Acute Respiratory symptoms. Mainspring of achieving this present study results ;probably since respiratory environment is suitable for Chlamydia pneumoniae living and growth and exists as an weak and Fugacious body when outside of respiratory system or it may be killed convenient in blood circulation because of more availability for humoral and cellular immune system as immunoglobulin and antibodies,

complements and circulating l granulocyte, lymphocyte, monocyte and also because of this organism has Intracellular growth thus due to Invocation to circulating system immediate would be killed. Because all of this reasons we could not discover Chlamydia pneumoniae in serum with PCR assay. The other studies that curried out in multiple region of the world discovery percentage and prevalence of Chlamydia pneumoniae from serum sample were a little or negative. In one study that has done via German nation-wide competence network CAPNETZ, Prevalence of Chlamydia pneumoniae between all etiologies of pneumoniae was lower than one percents. Thus be low because of etiology and it is role in create pneumonia. Thus in a outbreak of Acute respiratory syndromes even if we evaluate patients with high sensitive and specific assay ;should be expecte that prevalence of Chlamydia pneumoniae has been before 1 percent (13, 14).

Table 4. Absolute, relative and cumulative frequency of demographic, clinical symptoms and past history of patients

Variable	Absolute frequency (number)	Relative frequency (percent)	Cumulative Frequency (percent)
Sex Male Female	 49 55	 (1/47) (9/52)	 (1/47) (100)
Age 29 – 10 49 – 30 69 – 50 89 – 70	 50 33 17 4	 (1/48) (7/31) (3/16) (8/3)	 (1/48) (8/79) (2/96) (100)
Smoking Yes No	 20 80	 (2/19) (8/80)	 (2/19) (100)
Opium addiction Yes No	 6 98	 (8/5) (2/94)	 (8/5) (100)
Corticosteroid Yes No	 40 64	 (5/38) (5/61)	 (5/38) (100)
Previous pneumonia Yes No	 15 89	 (4/14) (6/85)	 (4/14) (100)
Underling respiratory diseases No Asthma Bronchithis Asthma & bronchithis	 99 2 1 2	 (2/95) (9/1) (1) (9/1)	 (2/95) (1/97) (1/98) (100)

In one study that has performed via Kaul & colleagues they characterized the cell population within peripheral blood mononuclear cells (PBMCs) that harbor *C pneumoniae* DNA. Adherent and nonadherent PBMCs from 28 patients with coronary artery disease (CAD) and 19 healthy blood donors have been evaluated for the presence of *C pneumoniae* DNA by touchdown nested polymerase chain reaction (nPCR). Of the 28 patients, 10 (36%) had detectable PCR product in their nonadherent and 3 (10%) in their adherent PBMC population.–specific PCR results have been positive for 5 of 19 (26%) healthy blood donors.they have detected *C pneumoniae* only in the nonadherent cell population among this group of individuals by PCR (15). Results of this study has indicated that cellular blood samples is preferable to serum blood samples for Chlamydia pneumoniae detection by PCR alike to our study. In Lochindarat and colleagues study from 24 patients with 5 – 15 years old only 17/5% has had Atypical pneumonia and 2/5% of them were positive for Chlamydia pneumoniae (16), But this study has presumed on nasal Discharged and not serum samples.

In Verkooyen and colleagues study, they have been discovered Chlamydia pneumoniae With PCR;

20% (31) of 156 community Acquired pneumonia samples (17). In this study 23 (25%) was positive for Chlamydia pneumoniae that is surprising because of serology is more sensitive to PCR usually.

In one study in Italia from 93 IHD patient, 24 (25/8%) and from 42 healthy individual 2 (4/8%) has had positive PCR for Chlamydia pneumoniae, But 76% of IHD patient and 42% of healthy Individual has had serologic positive for that (18).

In a study that carried out via Birkebaek and colleagues in Denmark from 201 patients with chronic cough up to 6 weeks and 106 healthy Individual. Only 9 (4%) patients and (1%) one health has had serologic finding and 3 of 9 patients were PCR positive for Chlamydia pneumoniae (19).

In Sudan one study carried cut on sputum discharge, 85 children with Respiratory symptom and 93 health child that 38 (5/7%) of health have had positive Chlamydia pneumoniae PCR (20). In A American Study that carried out on 28 patient with CAD and 19 health Individuals, while 10 (36%) of CAD patient and 5(29%) of healthy Individuals had positive PCR for Chlamydia pneumoniae (21).

Due to up and our study advice in future this study carry out on sputum and other upper or lower respiratory discharge then should be concomitant with a primary serologic screening or presume on peripheral blood mononuclear cells (PBMCs) for Chlamydia pneumoniae PCR detection, and since one of the most of etiology of atypical pneumonia and respiratory infection are viruses (22, 23). Thus for manage procure the better results we should be perform the other study with more sample that accompanied with accurate selection and screening primary patients that have never reception any antibiotics especially macrolides and fluoroquinolones, 3 months before referring.

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