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Comparisons detection of *Chlamydia pneumoniae* by Polymerase Chain Reaction and Serology in Khartoum state

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Abstract:

Background: *Chlamydophila pneumoniae (C. pneumoniae) is an obligate intracellular human pathogen, causing acute and chronic infections of the upper and lower respiratory tract. Pneumonia and bronchitis are the most common clinical manifestations of C. pneumoniae infection; approximately 10% - 20% of cases of community-acquired atypical pneumoniae (CAAP) are associated with C. pneumoniae.*

Objectives: *The objective of this study was to detection the causative agent of CAAP by molecular and serological methods in Khartoum state hospitals.*

Methods: ***F****our hundred patients with symptoms of pneumonia were enrolled in this study. The patients attended different Chest Units in Khartoum State. Blood and sputum were collected from each patient. Serum was separated from blood by centrifugation while genomic DNA was extracted from sputum specimen by bacterial preparation kit. To detect C. pneumoniae in the two types of specimens,*

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the former was tested by immunofluorescence test IIFT (IgM) and the later investigated by PCR technique.

Results: *Of the four hundred patients enrolled in this study 242(60.5 %) were males and 158(39.5%) females. The mean age of the patients was 42.1 years. The disease was more prevalent among males than females, however the relationship between C. pneumoniae and gender was insignificant (p=0.119).there a significant relation between C. pneumoniae and age groups (p<0.003). Adoption of immunofluorescence test and PCR technique revealed that 43(10.75%) and 69(17.25%) patients were infected with C. pneumoniae respectively. The results of serological test and PCR technique were statistically significant (p<0.000).*

Conclusion: *PCR the beast and accurate than conventional serological method especially for fastidious microorganism i.e. C. pneumoniae it is the one of the main contributing agent in Khartoum state.*

Key words: Atypical bacterial pneumonia, Serological IIFA, *Chlamydia pneumoniae*, PCR.

INTRODUCTION:

C. pneumoniae is a pathogenic bacterium responsible for respiratory tract infections (1). *C. pneumoniae* is oblige, intracellular, gram-negative bacterium that has been related with a big variety of acute and chronic diseases. Respiratory infection with *C. pneumoniae* occurs wide-reaching and in all age groups (2). It causes acute respiratory disease, mainly atypical pneumonia. High frequency of precedent infection with *C. pneumoniae* have been found on the grow countries that may has a accountability in pathogenesis of coronary heart disease (3), coronary heart disease is the most widespread type of heart disease that remains a major cause of morbidity and mortality in the industrialized world and become growing in developing countries (4). Diagnosis of infections caused by *C. pneumoniae*

can be done by immunostaining or separation and culturing of the organism (5). But *C. pneumoniae* require intracellular bacterium that must be cultured within eukaryotic host cells, this culture remains complicated, time-consuming, and insensitive (6). The microimmunofluorescence (IIFA) test is an indirect fluorescent antibody test that measures antibodies to epitope in the cell wall of *C. pneumoniae* elementary bodies quantitatively; time overriding and requires qualified personnel for reading of the slides. In addition the specificity of IIFA has been questioned, as cross-reactions among the major outer membrane proteins of different *Chlamydia* species were reported (7, 8). PCR has been described to be fast, sensitive, and specific technique for recognition of such an organism (9). The current study aimed to make a diagnosis of *C. pneumoniae* infection by two techniques (PCR techniques for detection of 16SrRNA gene *C. pneumoniae* and indirect microimmunofluorescence technique), compare between them to determine which was more sensitive more specific and more accurate.

MATERIALS AND METHODS:

Microbiology: Local institution (College ethical committee) review board approval was obtained for this study. Consent was obtained verbally from each patient. All information regarding risk factors was explained to all patients under. The study included 400 Sudanese patients with atypical pneumonia attended outpatients Chest Unit at Omdurman Teaching Hospital, AL-Shaab Teaching Hospital, Bahry Teaching Hospital and Abu Anja Hospital. 242(60.5%) of the patient were male and 158(39.5%) females. The patient's ages ranged from 13 to 91years. Sputum and Blood specimens were collected and transferred to the laboratory under the standard conditions

following the Guideline of National Committee for Clinical Laboratories Standards (NCCLS).

Serological test: Sera were separated from blood by centrifugation at 3000rpm for five minutes. The obtained sera were maintained in new sterile containers. Serological tests were done by Anti- *C. pneumoniae* immunofluorescence test IIFT (IgM) (Euroimmun, Germany) for *C. pneumoniae*. The procedure was carried out according to instructions of manufacturer.

PCR: DNA extraction was done by bacterial DNA preparation kit (Jena Bioscience. Germany). The extraction was carried out according to the manufacturer's instructions. Briefly 1 ml of sputum was transferred to 1.5 ml micro-tube, centrifuged at 15,000 rpm for 1 min. The supernatant was discarded. The pellet was re-suspended at 300 µl of Cell Lyses Solution. 1.5 µl of RNase a Solution was added and mixed gently by inverting, than Incubated at 37 °C for 15-30 minutes and cool in ice for 1 minute. 100 µl of Protein Precipitation Solution was added and vortexed vigorously for 20-30 seconds, the mixture was centrifuged at 15,000 rpm for 5 minutes. The supernatant was transferred to a clean 1.5 ml micro-tube containing 300 µl Isopropanol >99 %, mixed well by inverting gently for 1 minute and centrifuged at 15,000 rpm for 1 min. The supernatant was discarded on a clean absorbent paper, 500 µl washing buffer was added and invert the tube several times to wash the DNA pellet and centrifuged at 15,000 rpm for 1 minute. The ethanol was discarded carefully, air dried at room temperature for 10-15 minutes. At the end 50-100 µl of DNA Hydration Solution was added to the dried DNA pellets, incubated at 65 °C for 60 minutes. The obtained DNA was stored at -20°C until used.

Conventional PCR

The experimental DNA using Maxime premix kit (I-Taq) (Introgen Korea), positive control and a negative controls, 2µl DNA was added to PCR tube and the following solutions were placed in a total volume of 20 µl: 10X Taq buffer, 2.5 mM 4dNTP stock (final concentration 200 µmol), 10 pmol/µl primer F, 10 pmol/µl primer R, 100ng of genomic DNA template, $MgCl_2$ (final concentration 1.5µm), H_2O (up to the total volume of 20µl) and 2.5u Taq Polymerase. PCR amplification was performed using the following primers previously described by Jafer *et al*., (2013) for *C. pneumoniae* (Table 1).

Table 1. Primers and PCR products of 16SrRNA gene of *C. pneumoniae*

Primer specify	Primers	Primer pair Séquence (5' ---3')	Product size(bp)	References
C. pneumoniae	F	5-TGACAACTGTAGAAATACAGC-3	463bp	(10)
	R	5- CGCCTCTCTCCTATAAAT--3		

Cycling conditions was as follows: Initial denaturation step at 94°C for 5-minutes, 30 cycles each at 95°C for 30 sec, at 55°C for 30 seconds and 72°C for 30 seconds followed by at 5 minutes hold at 72°C. PCR success was examined on 1.5% agarose electrophoresis stained with Ethidium bromide.

Statistical analysis

Data were recorded and then analyzed using chi-square test by SPSS. All tests were two-tailed and a P-value of <0.05 was considered statistically significant.

RESULTS:

Out of four hundred screened patients 242(60.5 %) were males and 158(39.5%) females. The mean age of the patients was 42.1 years (range 13-91 years). They categorized in to four teaching

hospitals in Khartoum stat. 30, 58, 208, 104 from Omdurman Chest Unit, Abu Anja, AL-Shaab and Bahry respectively. Serum specimens investigated by immunofluorescence test 43(10.75%) were positive. PCR technique showed 69(17.25%) of 16SrRNA gene *C. pneumoniae* the relationship between serological test and PCR statistically significant (p<0.000) (Table 2) (fig 1, 2). Relation between hospital and *C. pneumoniae* found insignificant (p= 0.211) (Table 3). The prevalence in males was more than females but the relationship between *C. pneumoniae* and gender was insignificant (p=0.119) (Table 4). There a significant relation between *C. pneumoniae* and age groups (p<0.003) (Table 5).

Table 2: Relationship between *C. pneumoniae* PCR and Serology

***C. pneumoniae* PCR**		**PCR**		**Total**
		Positive	**Negative**	
Positive	Count % of Total	43 10.75%	26 6.5%	69 17.25%
Negative	Count % of Total	0 0%	330 82.5%	331 82.75%
Total	Count % of Total	43 10.75%	357 89.25%	400 100.0%

P= 0.000

Table 3: Relationship between hospitals and *C. pneumonia*

Hospital	***C. pneumoniae***		**Total**
	Positive	**Negative**	
Refer clinic Omdurman	8 (2.0%)	22 (5.5%)	30 (7.5%)
Abu anja	11 (2.8%)	47 (11.8%)	58 (14.5%)
Al shaab	38 (9.5%)	170 (42.4%)	208 (52.0%)
Bahry	12 (3.0%)	92 (23.0%)	104 (26.0%)
Total	69 (17.3%)	331 (82.7%)	400 (100.0%)

P= 0.211

Table 4: Relationship between Gender and *C. pneumoniae*

Gender	*Chlamydia pneumoniae*		Total
	Positive	Negative	
Male	48 12.0%	197 49.3%	245 61.3%
Female	21 5.3%	134 33.4%	155 38.7%
Total	69 17.3	331 82.7	400 100.0%

P= 0.119

Table 5: Relationship between age group and *C. pneumoniae*

Age group (year)	*C. pneumoniae*		Total
	Positive	Negative	
1-30	31 (7.8%)	91 (22.8%)	122 (30.5%)
31-60	31 (7.8%)	187 (46.8%)	218 (54.5%)
61-91	7 (1.8%)	53 (13.3%)	60 (15.0%)
Total	69 (17.3%)	331 (82.7%)	400 (100.0%)

P= 0.003

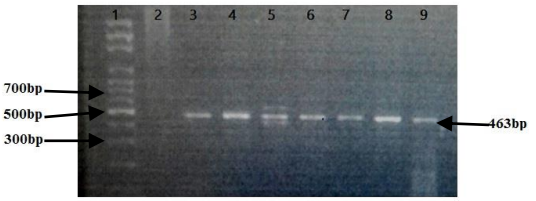

Fig 1: **PCR products of 16SrRNA gene of *C. pneumoniae***

Figure 2. 1.5 % agarose gel electrophoresis of *C. pneumoniae* by PCR and they have lane (1) M. Mw 100 – 1000 bp fragments – lane (2) control negative, lane (3) controls Positive. The

pictorial showed all (6) isolates (4, 5, 6, 7, 8, 9), with a band typical in size (463bp) which are positive for 16SrRNA gene.

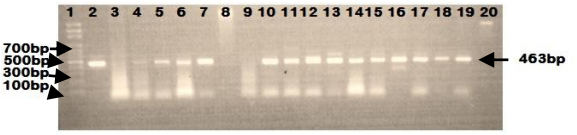

Fig 2. PCR products of .16SrRNA gene of *C. pneumoniae*

Figure 2. 1.5 % agarose gel electrophoresis of *C. pneumoniae* by PCR and they have lane (1) M. Mw 100 – 1000 bp fragments – lane (2) controls Positive. The pictorial showed all (14) isolates (4, 5, 6, 7, 10- 19), with a band typical in size (463bp) which are positive for 16SrRNA gene, (3) negative control, (8, 9, 20) isolates are negative.

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DISCUSSION:

The predominance of *C. pneumoniae* in Community Acquired pneumonia is high percent and there is a large association between *C. pneumoniae* and coronary vessels (11). There is a numeral of kinds of analytical assays For *C. pneumoniae* as: Serology with IFA, PCR and Culture (6, 12). Culture of *Chlamydia* is very difficult because of requirements to eukaryotic cells and it is a non responsive test. For that reason one of the best assay for differentiate of *C. pneumoniae* is PCR; it is available and high accurate (13). Hence were carried out this study for determine *C. pneumoniae* in patients with

suspected atypical pneumonia and were compared between two methods. The serological test by IIFA showing 43(10.75%) and PCR technique showing 69(17.25%) of 16SrRNA gene and the serology sensitivity 62.3% and specificity 92.7% compare with PCR its represent the PCR is more sensitive and specific than serology were this results are agreement with several studies showing the PCR percentage is more than serology, Mohammad from Iran found Positive PCR detected in 19.6% (10/51) of cases and Positive IgM detected in 9.8% (5/51)(14), Melanie showing 54 (33%) specimens were positive for the presence of *C. pneumoniae* DNA Chlamydial antibodies were detected by MIF 43 (24%) of the patients tested(15). Cheuk results viewing the PCR more sensitive than MIF serology test (16), Nele study presentation CAP was caused by *C. pneumoniae* in 5/546 cases (0.9%). Antibody testing by microimmunofluorescence was done in 376 of 546 patients, all patients were negative for IgM antibodies and the conclusion PCR is more sensitive technique (17), Hem from India showing 29.6% (27/91) patients were positive for *C. pneumoniae* using nested PCR compared with *C. pneumoniae* specific IgA, IgA IgG and IgG antibodies 11(12%) were IgA positive, 13(14.2%), IgA IgG positive and only (1.1%) was IgG positive (18), Ali viewing Serological acute infection for *C. pneumoniae* was not detected with patients positive PCR results (19). Our results disagreement with Zheng that the sensitivity, specificity, and concordance rate of microimmunofluorescence and ELISA tests more specific were compared with those of polymerase chain reaction (PCR) (20). That because used a novel monoclonal antibodies against a recombinant protein corresponding to the immunodominant area of chlamydial protease-like activity factor from *C. pneumoniae*. On our results showing a high prevalence of *C. pneumoniae* because that a large number of specimens from Alshaab hospital (cardiac and chest infection hospital), most of outpatients may have complication chest and cardiac infections

because chlamydial persistent form may endure for a long time inside host cells, since it is capable of escape the host immune response leading to a chronic inflammatory state in the vascular wall (21, 22).

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