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Detection of *Mycoplasma pneumoniae* in Khartoum State Hospitals: A cross-sectional Study

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Abstract:

Background and objectives: *Mycoplasma pneumoniae (M. pneumoniae) continues to be a major cause of human respiratory infections such as atypical pneumonia, mainly in children and adults. The objectives of this study were to detect M. pneumoniae in patients with pneumonia as well as to determine the prevalence of the disease in Khartoum State hospitals.*

Methods: *400 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 DNA preparation Kit. To detect M. pneumoniae in the two types of specimens, the former was tested by ELISA and the later was 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 M. pneumoniae and gender was insignificant (p=0.346). On the other hand, significant relationship was found between C. pneumoniae and age groups (p<0.003). Adoption of ELISA test and PCR technique revealed that 32(8.0%) and 49(12.25%) patients were infected with M. pneumoniae respectively. The results of serological test and PCR technique were statistically significant (p<0.000).*

***Conclusion:** The PCR is accurate, rapid and specific diagnostic method for M. pneumoniae than the serological method. However, a combination of the two methods is necessary for the detection of M. pneumoniae infections to reduce morbidity and mortality due to this organism.*

Key words: Atypical bacterial pneumonia, Serological ELISA, Mycoplasma pneumoniae, PCR.

INTRODUCTION:

M. pneumoniae, a frequent cause of respiratory tract infections that is transmitted from person to person through aerosolization. *M. pneumoniae* is one of the most prevalent causes for respiratory tract infections worldwide extra pulmonary manifestations are present in up to 25% of all infected persons [(1)]. The infection occurs in all age groups, but older children and young adults are disturbing at a higher rate than other age groups. Approximately 10% of the cases of community-acquired pneumonia that occurs in endemically, and up to 50% of the cases that occur in epidemic periods are caused by *M. pneumoniae*. Clinical manifestations range from mild cases of tracheobronchitis to severe atypical pneumonia and can be following by a wide range of extra pulmonary complications [(2)]. *M. pneumoniae* is a cell wall-less bacterial pathogen of the individual respiratory tract [(1)]. The unfinished

biosynthetic capabilities of *mycoplasma* are usually explained by their growth as obligate parasites of diverse eukaryotic hosts [3]. Colonization of the host respiratory epithelium by *M. pneumoniae* requires gliding motility [4] which force facilitates contact to receptors on the host cell surface and succeeding lateral spread. The gliding apparatus of *M. pneumoniae* is a polar terminal structure [5] that also functions in cell division [6] and sticking to host receptors[3, 4]. The lack of nearly all anabolic pathways known from more comprehensive organisms highly facilitates the straight relationship of extracellular nutrient reduction to cellular processes [7]. They require of rapid and accurate investigative laboratory tests to detect *M. pneumoniae* directly or the serologic response it elicits has hampered understanding of the epidemiology and contributed to the unawareness of the potential clinical implication of this common pathogen[8]. The identification of *M. pneumoniae* pneumonia still relies on traditional methods of culture and serology, both of which fail to congregate the criteria of a proper diagnostic test. Culture is expensive, time consuming and laboratory demanding and is successful in only 23%–64% of serologically diagnosed cases. Serological methods are easier to perform but are generally non-specific with revere to IgM response, insensitive and demonstration [9]. Polymerase chain reaction (PCR), a rather recent technique, has been applied for the sensitive and specific detection of a variety of infectious agents including the Mycoplasma spp [10, 11, 12]. This study was essentially designed to detect *M. pneumoniae* in patients with pneumonia as well as to determine the prevalence of the disease in Khartoum State hospitals using PCR technique and ELISA serological test.

MATERIALS AND METHODS:

Local institutional (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. The study included 400 Sudanese patients with atypical pneumonia attended outpatients Chest Units at Omdurman Teaching Hospital, AL-Shaab Teaching Hospital, Bahry Teaching Hospital and Abu Anja Teaching Hospital. 242(60.5%) of the patients were males and 158(39.5%) females. The patients' ages ranged from 13 to 91 years. Sputum and Blood specimens were collected and transferred to the laboratory under the standard conditions following the Guidelines of National Committee for Clinical Laboratories Standards (NCCLS).

Serological test:

Sera were separated from blood by centrifugation at 300rpm for five minutes. The obtained sera were maintained in new sterile containers. Serological tests were done by ELISA IgM (Euroimmun, Germany) for *M. 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 a 1.5 ml micro-tube, centrifuged at 15,000 rpm for 1 min. The supernatant was discarded. The pellets were re-suspended in 300 µl of Cell Lyses Solution. 1.5 µl of RNase solution was added and mixed gently by inverting, then incubated at 37 °C for 15-30 minutes and cool on 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 minute. The supernatant was discarded on a clean absorbent paper, 500 µl of 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 discarded; 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 PCR premix kit (I-Taq) (Introgen Korea), positive control and negative controls, 2µl 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, 100 ng of genomic DNA template, $MgCl_2$ (final concentration 1.5µm), H_2O (up to the total volume of 20µl) and 2.5uu Taq Polymerase. PCR amplification was performed using the following primers previously described by Zibo *et al.,* (2015) for *M. pneumoniae* (Table 1):

Table 1. Primers of *M. pneumonia*e

Primer specify	Primer	Primer pair Séquence (5' ---3')	Size	References
M.pneumoniae	FR	5-AAGGACCTGCAAGGGTTCGT-3 5-CTCTAGCCATTACCTGCTAA -3	277bp	[13]

Cycling conditions were as follows; initial denaturation at 95°C for 5 minutes, 30 cycles each at 95°C for 30 seconds, at 54°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 data analysis:

Data were recorded and then were 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. Median age of the patients was 42.1 years (range 13–91 years). They categorized into four age groups. The patients were attended to four teaching hospitals in Khartoum State. Sputa were collected from patients in each hospital as follows; 30, 58, 208, 104 from Omdurman, Abu Anja, Al shaab, and Bahry respectively. Serum specimens investigated by ELISA test revealed 32(8%) were positive the ratio more than (1.1). The negative specimens gave the ratio (<0.8), but there is no specimen in borderline. PCR technique showed 49(12.25%) of 16SrRNA gene *M. pneumoniae*, the relation between serological test and PCR statistically significant (*P*=0.000) (Fig 1).

Relation between hospitals and *M. pneumoniae* found insignificant (*P*=0.192 (Table 2). The prevalence in males was more than females but the relationship between *M. pneumoniae* and gender was found insignificant (*P*= 0.346) (Table 3). There is a significant relation between *M. pneumoniae* and age groups (*P*<0.003) (Table 4).

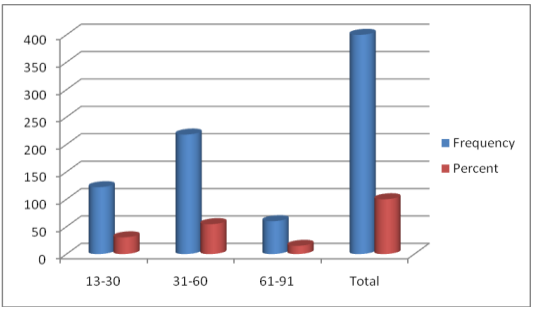

Fig 1. Distribution the age groups of *M. pneumoniae*

Table 2. Relationship between hospitals and *M. pneumoniae*

Hospitals	*M. pneumoniae*		Total
	Positive	Negative	
Refer clinic	3	27	30
Omdurman	0.8%	6.8%	7.5%
Abu anja	6	52	58
	1.5%	13.0%	14.5%
Al shaab	21	187	208
	5.3%	46.7%	52.0%
Bahry	19	85	104
	4.8%	21.3%	26.0%
Total	49	351	400
	12.3%	87.7%	100.0%

P= 0.192

Table 3. Relationship between Gender and *M. pneumoniae*

Gender	*M. pneumoniae*		Total
	Positive	Negative	
Male	27	218	245
	6.8%	54.4%	61.3%
Female	22	133	155
	5.5%	33.3%	38.7%
Total	49	351	400
	12.3	87.7	100.0%

P= 0.346

Table 5. Relationship between age group and *M. pneumoniae*

Age group	*M. pneumoniae*		Total
	Positive	Negative	
1-30	10	112	122
	2.5%	28.0%	30.5%
31-60	25	193	218
	6.3%	48.3%	54.5%
61-91	14	46	60
	3.5%	11.5%	15.0%
Total	49	351	400
	12.3%	87.7%	100.0%

P= 0.002

Table 6. Relationship between *M. pneumoniae* PCR and Serology

Mycoplasma pneumoniae PCR		*Mycoplasma* PCR		Total
		Positive	Negative	
Positive	Count % of Total	32 8.0%	17 4.3%	49 12.3%
Negative	Count % of Total	0 0%	351 87.7%	351 87.7
Total	Count % of Total	32 8.0%	368 92.0%	400 100.0%

P= 0.000

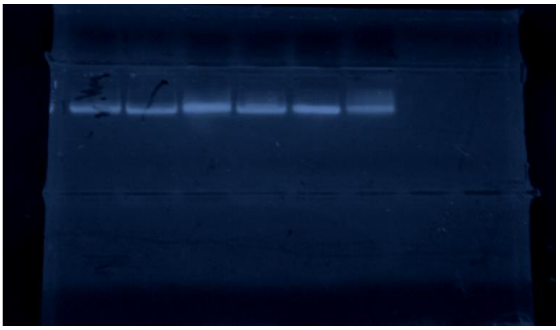

Fig 2. Analysis of the extracted genomic DNA in 1.0% agarose gel with 1X TBE buffer

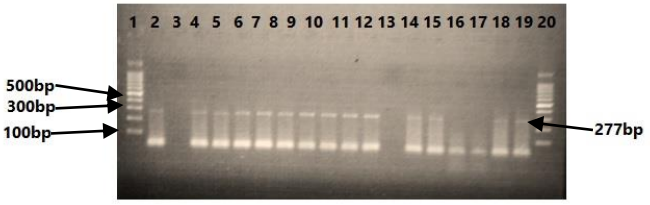

Fig 3. PCR products of 16SrRNA gene of *M. pneumonia*e

Figure 2. 1.5 % agarose gel electrophoresis of *Mycoplasma pneumonia*e by PCR and they have land one M. Mw 100 – 1000 bp fragments – lane two controls Positive , land three negative control. The a pictorial showed all 13

isolates (4-12,14,15,18,19), with a band typical in size (277bp) which are positive for 16SrRNA gene, (13,16,17) isolates negative 16SrRNA gene and land (20) M. Mw 100 – 1000 bp fragments.

Key. First lane; Marker; second land, Positive control, Lanes, Third land negative control (4,5,6,7,8,9,10,11,12,14,15,18,19) were 16SrRNA gene, lane (13,16,17) negative, lane (20) M. Mw 100 – 1000 bp fragments.

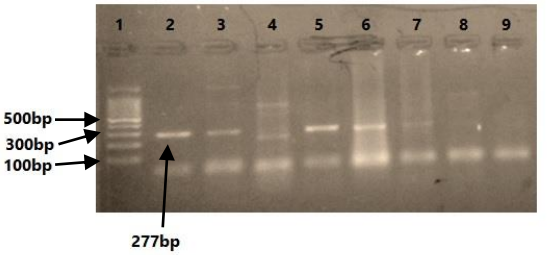

Fig 4. PCR products of 16SrRNA gene of *M. pneumonia*e

Figure 2. 1.5 % agarose gel electrophoresis of *Mycoplasma pneumonia*e by PCR and they have land one M. Mw 100 – 1000 bp fragments – lane two controls Positive. The a pictorial showed all 4 isolates (3,5,6,7), with a band typical in size (277bp) which are positive for 16SrRNA gene, (4,8) isolates non specific band , (9) negative control.

Key: First lane; Marker; second land, Positive control, Lanes (3, 5, 6, 7) were 16SrRNA gene, lane (4,8) non specific bands, lane (9) negative control.

DISCUSSION:

The gold standard method for the diagnosis of *M. pneumoniae* as an agent for atypical pneumonia has been culture. Researchers stated that PCR is more accurate as a diagnostic test than conventional techniques [14, 15].

The present study was designed essentially to detect *M. pneumoniae* in patients with atypical pneumonia. The organism has been frequently observed in patients suffering with respiratory illness and is also reported to be related with acute exacerbation of bronchial asthma and chronic obstructive pulmonary disease [16], stroke [17], polyarthritis [18], acute respiratory distress syndrome [19] Guillain-Barre syndrome [20]

and coronary artery diseases [21]**.** The PCR technique in our study revealed that 49(12.25%) of the enrolled patients were positive for *M. pneumoniae*. This result is in agreement with that reported by Roger and his co-workers in Germany (12.3%) (2). On the other hand, serological test showed only 32(8.0%) of the same group of patients were positive *M. pneumoniae*. This result in line with several studies such as Jiuxin and his colleagues in China (7.4%, 28.6% and 1.45) compare with PCR (40.7%, 50% and 3.63) on three months, Kate and others approved that 12 (11.3%) were positive by all the molecular methods whereas serology with acute sample and convalescent samples detected only 6 (5.6%) and 9 (8.5%), Bineeta *et al*., in India reported that PCR positive for *M. pneumoniae* infection in 18 (24%) compared to serological evidence of *M. pneumoniae* infection 16(21.3%) [22, 23, 24]. The reason for the differences between PCR and serological methods may be due to the fact that serological methods usually lack adequate sensitivity in the acute phase of the disease, and perfect diagnosis with convalescent phase samples is often made many days after the beginning of disease [25], Sensitivity and specificity values are between 55 and 100%, depending on the serological methods used and the patient population tested [26]. The PCR technique has been found to be more sensitive and rapid compared to serological diagnostic tests [27].

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