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Molecular and serological detection of *Legionella pneumophila* among Sudanese patients

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Abstract:

Background: *Legionella pneumophila (L. pneumophila) is a severely environmental and opportunistic pathogen that can cause strict pneumonia after inhalation of aerosols with enough bacterial lots, bacterium is able to surviving an extreme range of environmental conditions.*

Objectives: *The aim of this study was to detection of Legionella pneumophila by molecular and serological methods among Sudanese patients.*

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*

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preparation kit. To detect L. pneumophila in the two types of specimens, the former was tested by immunofluorescence test IIFT (IgG, IgAGM) 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 L. pneumophila and gender was insignificant (p=0.119). There a significant relation between L. pneumophila and age groups (p<0.003). Adoption of immunofluorescence test and PCR technique revealed that 50(12.5%) and 57(14.25%) patients were infected with L. pneumophila respectively. The results of serological test and PCR technique were statistically significant (p<0.000).*

Conclusion: *Combining both PCR and serodiagnosis (based specific IgG, IgAGM antibody) may be a more reliable diagnostic of L. pneumophila in Sudanese patients.*

Key words: Atypical bacterial pneumonia, *L. pneumophila*, Serological IIFA (IgG, IgAGM) and *PCR*

INTRODUCTION:

The Legionellaceae were not documented until 1976 when hazardous outbreak of pneumonia occurred in Philadelphia at an American legion reunion. Thirty four of the 221 people who became ailing after disclosure died within the first few weeks after the convention (1). *L. pneumophila* is the contributory cause of severe serious pneumonia (Legionnaires' disease), commonly requiring hospitalization (2). *L. pneumophila* is a fastidious Gram-negative bacterium that resides in water environments. They have been recognized as a significant cause of severe community acquired pneumonia (CAP) (3). The symptoms of Legionnaire's disease, headache, diarrhea, fever, chills, abdominal pain and myalgias as well as a non-productive cough (4). *L. pneumophila* is valuable more than 90% of the legionary, which is finely tuned respiratory disease. Serogroup

1 and 6 be the causes of two thirds of cases of *Legionella* disease (5). Mortality rate in old and immunocompromised patients with *L. pneumonia* may be further more 30% (6). Allowing for medical signs and symptoms of infection, *L. pneumonia* cannot be differentiated from other pneumonia (7). Suitable diagnosis and treatment of disease is successful in reducing the people rate. Epidemiological conclusion indicates that this bacterium is transmitted through aerosols out of contaminated water sources and involves the respiratory system. Hospital atmosphere as enlargement area and community at risk of aerosol spread are latent predisposing factors for growth and increase of this causative agent. *Legionella* are generally dispersed in natural and man-made water sources (8). Culture technique like other bacteria is the gold standard for recognition of these bacteria; the use of this process is limited for the diagnosis of infection in patients, because the bacterium requirements at least seven days to come into observation colonies (9). Serological tests for identification of *Legionella* infections are expensive epidemiological instrument, but not useful for fast recognition of acute cases of Legionnaires' disease (10). The use of molecular methods in identification of Legionnaires' disease has very high investigative sensitivity, specificity and rapidity (11). Diagnosis can be confirmed via detection of *L. pneumophila*, often inaccessible from respiratory secretions, by culturing, urine antigen tests, immunofluorescent staining, PCR or serologic tests (12). Still though the outbreak in a lot of countries no studies were agreed out in Sudan to determine the frequency of *L. pneumophila* as a cause of community acquired pneumonia.

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- *L. pneumophila* immunofluorescence test IIFT (IgG, IgAGM) (Euroimmun, Germany) for *L. pneumophila*. 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 *L. pneumophila* (Table 1).

Table (1): Primers and PCR products of *MIP* gene of *L. pneumophila*

Primer specify	Primers	Primer pair Séquence (5' ---3')	Product size(bp)	References
Legionella. Pneumophila	FR	5-GCTACAGACAAGGATAAGTTG-3 5-GTTTTGTATGACTTTAATTCA -3	649bp	(13)

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 1minut and 72°C for 1 minute 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 50(12.5%) were positive. PCR technique showed 57(14.25%) of MIP gene *L. pneumophila* the relationship between serological test and PCR statistically significant ($p<0.000$) (Table 2) (fig 1, 2). Relation between hospital and d *L. pneumophila* found insignificant ($P= 0.301$) (Table 3). The prevalence in males was more than females but the relationship between *L. pneumophila* and gender was insignificant ($p=0.365$) (Table 4). There a significant relation between *L. pneumophila* and age groups ($p<0.002$) (Table 5).

Table 2: Relationship between Legionella pneumophila PCR and Serology

Legionella pneumophila PCR		Legionella PCR		Total
		Positive	Negative	
Positive	Count % of Total	50 12.5%	7 1.8%	57 14.3%
Negative	Count % of Total	0 0%	343 85.7%	343 85.7
Total	Count % of Total	50 12.5%	350 87.5%	400 100.0%

P-value= 0.000

Table 3. Relationship between hospitals and *L. pneumophila*

Hospital	***L. pneumophila***		**Total**
	Positive	**Negative**	
Omdurman Chest	7 (1.8%)	23 (5.8%)	30 (7.5%)
Abu anja	5 (1.3%)	53 (13.3%)	58 (14.5%)
Al shaab	31 (7.8%)	177 (44.2%)	208 (52.0%)
Bahry	14 (3.5%)	90 (22.5%)	104 (26.0%)
Total	57 (14.3%)	343 (85.7%)	400 (100.0%)

P= 0.301

Table 4: Relationship between Gender and *Legionella pneumophila*

Gender	*Legionella pneumophila*		Total
	Positive	Negative	
Male	38 9.5%	207 51.7%	245 61.3%
Female	19 4.8%	136 34.0%	155 38.7%
Total	57 14.3	343 85.7	400 100.0%

P-value= 0.365

Table 5: Relationship between age group and *L. pneumophila*

Age group	*L. pneumophila*		Total
	Positive	Negative	
1-30	12 (3.0%)	110 (27.5%)	122 (30.5%)
31-60	28 (7.0%)	190 (47.5%)	218 (54.5%)
61-91	17 (4.3%)	43 (10.8%)	60 (15.0%)
Total	57 (14.3%)	343 (85.7%)	400 (100.0%)

P= 0.002

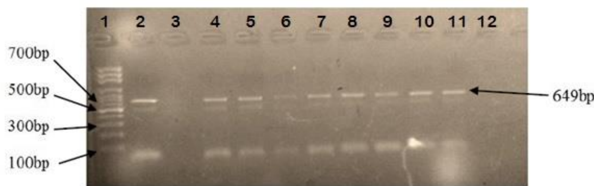

Fig 1: **PCR products of MIP gene of *L. pneumophila***

Fig 1. 1.5 % agarose gel electrophoresis of *L. Pneumophila* by PCR and they have land one M. Mw 100 – 1000 bp fragments – lane two controls Positive. The pictorial showed all (8) isolates (4, 5, 6, 7, 8, 9, 10, 11), with a band typical in size (649bp) which are positive for *MIP* gene, (3) negative control (12) isolates are negative.

Key. First lane; Marker; second land, Positive control, Lanes (4, 5, 6, 7, 8, 9, 10, 11) were *MIP* gene, lane (3) negative control (12) are negative.

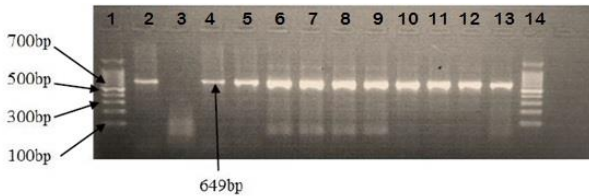

Fig 2: PCR products of MIP gene of *L. pneumophila*

Fig 2. 1.5 % agarose gel electrophoresis of *L. Pneumophila* by PCR and they have land one and (14) M. Mw 100 – 1000 bp fragments – lane two controls Positive. The pictorial showed all (10) isolates (4, 5, 6, 7, 8, 9, 10, 11, 12, 13), with a band typical in size (649bp) which are positive for *MIP* gene, (3) negative control.
Key. First lane and (14); Marker; second land, Positive control, Lanes (4, 5, 6, 7, 8, 9, 10, 11, 12, 13) were *MIP* gene, lane (3) negative control.

DISCUSSION:

The Atypical Pneumonia PCR Panel assay is able to detect the presence of any one of pathogens from a single respiratory specimen (14). The serological study for *Legionella* was a specific and accurate method which could be used to screen CAP patients in the absence of a culture facility (15). Our study of 400 serum specimens investigated by serological test gave positive 50(12.5%) IgM. Were DNA specimens the PCR technique showing 57(14.25%) of *MIP* gene *L. pneumophila.* This results are agreement and disagreement with previous studies, the result of PCR (14.25) was agreement with study done by Cloud total of 212 specimens examined by both culture and PCR for detection of *Legionella* spp, (14. 6%) samples positive by culture were also positive by PCR (16). Disagreement with Cianciotto 120 samples, while 57 samples (47.5%) had positive findings in real time PCR method (17), the

different may in number of specimens. Many studies showed that the use of molecular methods in diagnosis of infection caused by *L. pneumophila* has a great value because of its high specificity and rapid diagnosis of disease (18). The seroprevalence of our study (12.5%) is some agreement and disagreement with previous studies, total prevalence of *L. pneumophila* in the hospital samples of (19, 20, 21 and 22), 2.85%,13%, 63% and 46.66%, respectively In a study done on lower respiratory tract infections in Spain, 11% of the patients tested using IgG antibody detection had *Legionella* (23).

These investigations highlight large differences in the prevalence of *L. pneumophila*. This could be related to differences in the type of sample (bronchoalveolar lavage, hospital water, stool, blood, urine, and other clinical samples) tested, number of samples, method of sampling, history of patients (with and without smoking history), season of sampling, experimental methodology, geographical area, and climate differences in the areas where the samples were collected, Which would have differed between each study.

The reason for the different between PCR and serology because Serological methods lack sufficient sensitivity in the acute phase of the disease, an perfect diagnosis with restorative phase samples is often made many days after the beginning of disease (24), Sensitivity and specificity values are between 55 and 100%, depending on the serological method used and the patient people tested (25). PCR has been shown to offer the suppressed of increased sensitivity and rapidity compared to other diagnostic tests (26). In our study *L.pneumophila* increased in age group 31-60 statistically significant p=0.002 and this distribution agreement with the age distribution of the 71 cases of *L. pneumophila* showed that 45 (63. 3%) cases were between 30-70 years of age statistically significant (27). In conclusion with the added sophistication and modernization of amplification processes like multiplex PCR and real-time PCR, technology has enabled testing to be more proficient and

accurate. As with all molecular biology-based amplification methods, contamination and false positive results are always a risk. Good molecular biology practices in the laboratory and experience reduce this to a very low level (28).

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