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Molecular Detection of Legionella Pneumophila, Mycoplasma Pneumoniae and Chlamydia Pneumoniae among Sudanese Patients with Acute Respiratory Infections in Khartoum State, Sudan

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ABSTRACT

Background: *Bacterial infections affect humans causing mild to severe pneumoniae, particularly in immune-compromised individuals. The aim of this study was to detect and characterize Legionella pneumophila, Mycoplasma pneumoniae and Chlamydia pneumoniae among Sudanese patients in Khartoum state, Sudan.*

Materials and Methods: *Eighty throat swab specimens were collected from patients experiencing respiratory infection. C. pneumoniae, L. pneumophila, and M. pneumoniae were detected by multiplex polymerase chain reaction (PCR).*

Results: *Out of the eighty specimens tested from respiratory infection L. pneumophila was detected in 43(53.8%), M. pneumoniae in 14(17.5%) and C. pneumoniae in 9 (11.3%) using multiplex PCR. Mixed infections with two or three of these organisms constituted a high proportion of these infections (15%), while single infections with L. pneumophila, M. pneumoniae and C. pneumoniae represented 38.8%, 3.8 % and 5% of the infections respectively,*

Conclusions: *This is first report on molecular detection of Legionella pneumophila in Sudan. Multiplex PCR can offer a rapid method for detection of Legionella pneumophila, Mycoplasma pneumoniae and Chlamydia pneumoniae in patients with respiratory tract infection in a hospital setting in Sudan.*

Keywords: *Chlamydia pneumoniae, Legionella pneumophila, Mycoplasma pneumoniae, Legionella, Multiplex PCR, Respiratory infections, Sudan*

INTRODUCTION

Pneumoniae, which is caused by wide variety pathogens and is characterized by an infection of the lung parenchyma (13). Acute pneumonias are those with a recent and sudden onset, and are commonly classified into two groups, nosocomial pneumoniae and community-acquired pneumoniae (CAP). Nosocomial pneumonias are mostly acquired in the hospital setting and are typically caused by pathogens other than those that cause CAP (13).

Acute upper respiratory infections usually benign, transitory and self-limited, but it could be serious disease in children, young infants and the elderly (14). Infection of the lower respiratory tract, especially pneumoniae, can be severe or fatal. Atypical bacteria (*Mycoplasma, Legionella* and *Chlamydia*) are the dominant pathogens in lower respiratory infection (3). Management of CAP requires prompt diagnosis in order to initiate early proper antibiotic therapy based on the knowledge of the likely etiologic agent. On the other hand, the increase of resistant bacteria including CAP pathogens is a worldwide health problem (10).

Lower respiratory tract infections continue to be important cause of CAP and therapy is largely empirical because of the inability to determine the causative microorganisms of most patients by the time treatment is initiated (7).

Atypical pneumoniae is used to describe pneumoniae caused by atypical pathogens such as *Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophilia,* and

others. The clinical course among children infected with these pathogens is different from that of other bacterial or viral infection, as most of them progress slowly and have no specific symptoms. Many studies reported that *Mycoplasma pneumoniae* is the most common atypical pathogen found among children with CAP, followed by *Chlamydia pneumoniae* [1, 2].

The diagnosis of atypical bacteria using molecular methods such as PCR or real time PCR is faster than the classical microbiological identification techniques because most of the atypical bacteria grow either slowly or not at all in culture, leading to delays in detection and diagnosis (4, 8). Such PCR assays have lessened the importance of culture as means for detecting *M. pneumoniae, L. pneumophila* and *C. pneumoniae;* and allowed their rapid and timely diagnosis (9).

MATERIAL AND METHOD

Study Site

This is a descriptive cross-sectional study conducted in 80 patients. The samples were collected from 80 patients (50 males and 30 females) suspected of respiratory tract infection (running nose, fever, sore throat, cough), seen at the Emergency Departments in Khartoum Hospitals (Ibrahim Malik teaching Hospital and Khartoum teaching Hospital (Alshaab teaching Hospital), during April 2013 to January 2014.The enrolled patients were of different ages groups (up to 5, 5–25, and 25–55 years old),

Samples Collection

Throat swab samples were obtained from each patient by inserting sterile nylon swab (Regular Flocked swab, Copan Diagnostics Inc., Murrieta, Calif, USA) and rubbing the tonsils and the posterior wall of the pharynx. The collected samples were transported in an ice box on the same day of collection to the laboratory and stored at -20 °C till used.

DNA Extraction

DNA was extracted by using Qiagen DNA mini kit according to the protocol of the manufacturer (Qiagen, Germany), briefly, A 20 µL protease K was pipette into 1.5 mL microcentrifuge tube containing 200 µL of sample and 200 µL AL buffer was added. The samples were vortexed for 15 sec. and incubated at 56 °C for 10 min, then centrifuged briefly. A 200 µL ethanol (96–100%) was added to the tube, vortexed for 15s and briefly centrifuged to remove drops from the inside of the lid. The mixture was carefully applied to the QIAamp Mini spin column, in a 2 mL collection tube, centrifuged at 6,000 x g for 1 min. A 500 µL AW1 buffer was added to the column, centrifuged at (6,000 x g) for 1 min., 500 µL AW2 buffer was then added, centrifuged at full speed (20,000 x g) for 3 min. The QIAamp Mini spin column was then placed in a clean 1.5 mL micro-centrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was opened and 200 µL of AE buffer was added, incubated at room temperature for 1 min, and then centrifuged at (6,000 x g) for 1 min and stored at -20 °C until used.

Multiplex PCR

The multiplex PCR amplification primers were (the P1 gene, forward GTTTGCTGCTAACGAG TACGAG and Reverse GTAATCATCGTCTG ACTGCC for *Mycoplasma pneumoniae*, the mip gene forward CAATGGCTGCAACCGATGC, Reverse GGGATAACTTGTGAAACCTG for *Legionlla pneumophilia* and the PstJ gene forward CGGCTAGAAATCAATTATAAGAC TG, Reverse GGTGTGTTTCTAATACCTGTCC for *Chlamydia pneumoniae*); the expected size bands for the three genes were 360, 487, 283 BP respectively. The primers used in this study were validated by Erin *et al.*, 2005; it showed 100% sensitivity and specificity (11).

For the optimized multiplex PCR reaction amplification of *Mycoplasma, Legioella and chylamdia*, five ul of DNA extract was added to PCR premix (Maxime PCR premix kit (I-Tag), (Intron, Korea) containing I-Tag TM DNA polymerase, and dNTP mixture and reaction buffer. Two ul of primers and 13ul of distilled water were then added to PCR premix. PCR amplification was then performed using an (Techne, UK); denaturation was performed for 15 min at 95°C followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at 58°C for 90 sec., and primer extension at 72°C for 60 s, and a final product extension at 72°C for 7 min (11).The amplified PCR products were then subjected to agarose gel electrophoresis for 50 min at 120V using 1.5% agarose and IX TBE buffer with ethidium bromide. The gel was visualized using Gel Documentation System (UV solo TS, Analytik, Jena Company, Germany).

Result

Forty three (53.8%) out of eighty specimens showed PCR positivity for *L. pneumophila,*

14(17.5%) for *M. pneumoniae* 9 (11.3%) for *C. pneumoniae*. Singular infection with *L. pneumophila, M. pneumoniae* and *C. pneumoniae* were detected in 31(38.8%), 3(3.8%) and 4(5%) patients respectively. Dual infection caused by *Mycoplasma* + *Chlamydia*;

Table1. *Number of patients with Legionella, Chlamydia and Mycoplasma infections according to gender*

	Male	Female	Total	*P. value*
Legionella	18(22.5%)	13(16.2%)	31(38.8)	0.515
Chlamydia	1(1.2%)	2(2.5%)	3(3.8%)	0.596
Mycoplasma	3(3.8%)	1(1.2%)	4(5%)	0.287
Legionella + Mycoplasma + Chlamydia	3 (3.8%)	1(1.2%)	4 (5%)	0.648
Mycoplasma + Legionella	5 (6.2%)	1(1.2%)	6 (7.5%)	
Legionella + Chlamydia	1(1.2%)	1(1.2%)	2 (2.5%)	
Total	31(62%)	19(63.3%)	50 (%)	

P values > 0.05 is statistically insignificant

Mycoplasma + Legionella; and Legionella + Chlamydia was recorded in 0 (0%) 6(7.5%) and 2 (2.5%) patients respectively. Triple infections caused by *Legionella + Mycoplasma + Chlamydia* were recorded in only 4(5%) of the patients. Mixed infections recorded the prevalence rate (15%) and was higher in males 9(11.2%) than in females 3(3.8%), But no significant differences were discernible regarding the overall prevalence rates of these infections.

Based on age *L. pneumophila* were detected in 18(22.5%), 9(11.2%) and 4(5%) patients in the age groups <5, 5–25, and 25–55 years old; *M. pneumoniae* were detected in 3(3.8%), 1(1.2%) and 0(0%) patients in the age groups <5, 5–25, and 25–55 years old; *C. pneumoniae* was detected in 2(2.5%), 0(0%) and 1(1.2%) patients in the age groups <5, 5–25, and 25–55 years old respectively. The triple infection with *Legionella, Mycoplasma* and *Chlamydia* were detected in 3(3.8%), 1(1.2%) and 0(0%) patients in the age groups <5, 5–25, and 25–55 years old respectively.

The duel infection with *Mycoplasma + Legionella* were detected *in* 3(3.8%), 3(3.8%) and 0(0%) in the age groups <5, 5–25, and 25–55 years old respectively; and *Legionella + Chlamydia* were detected 1(1.2%), 0 (0%) and 1(1.2%)in the age groups <5, 5–25, and 25–55 years old respectively(**Table 2.**).However, no significant differences between age groups as regards prevalence of all infections were detected *P values* range (0.504 - 0.544).

Based on gender, *Legionella* infection was higher in males 18(22.5%) than females 13(16.2%) females; *Mycoplasma* was higher in males 18(22.5%) males than males 18(22.5%); *Chlamydia* was higher females2 (2.5%) than in males 1(1.2%), While mixed infections were higher in males 9(11.2%) than in females 3(3.8%) (Data not shown) **(Table1).**

Table2. *Number of patients with Bacterial Atypical pneumoniae according to age*

Age group in years	**Bacterial Atypical pneumoniae**					
	Legionella	***Mycoplasma***	***Chlamydia***	***Leg.+ Myco. + Chlam.***	***Mycoplasma + Legionella***	***Legionella + Chlamydia***
Less than 5	18(22.5%)	3(3.8%)	2(2.5%)	3(3.8%)	3(3.8%)	1(1.2%)
5-25	9(11.2%)	1(1.2%)	0	1(1.2%)	3(3.8%)	0
More than 25	4(5%)	0	1(1.2%)	0	0	1(1.2%)
Total	**31(38.8%)**	**4(5%)**	**3(3.8%)**	**4(5%)**	**6(7.5%)**	**2(2.5%)**

DISCUSSION

Atypical pathogens are one of most important causes of pneumoniae and the etiologic diagnosis of infections with atypical pathogens such as *C. pneumoniae, M. pneumoniae and L. pneumophila* remains difficult and requires a different approach for diagnosis. The present study aimed atmolecular detection for diagnosis of *Legionella pneumophila, Mycoplasma pneumoniae*, other *Mycoplasma spp* and *Chlamydia pneumoniae* among Sudanese patients with respiratory infections in Khartoum State, Sudan.

In Sudan, there has been no previous study of detection of atypical pneumoniae by using PCR but only 6.7% L. pneumophila *prevalence* was found using culture in environmental water sources by Mustafa *et al.*,(2013) and detection of legionella IgG antibodies (22.7%) by Rabih *et al.*,(2014).

In this study, the acute respiratory infectionscaused by *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* as a single or mixed infection constituted, 62.5% of all of the positive specimens comprising 38.8%of singular infection, 10% as dual infection and 5% as a triple infection. (Table1) (Data not shown).

In Saudi Arabia, the prevalence range was 37% of the total combined infection with *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila*; the single infection with *M. pneumoniae, C. pneumoniae, L. pneumophila* was 31.5%, 27.5% and 20% respectively. Dual infection caused by Mycoplasma + Chlamydia; Mycoplasma + Legionella; and Legionella + Chlamydia was 24%, 20% and 15% respectively. Triple agent infection caused by Legionella + Mycoplasma + Chlamydia was 17.5%, indicating higher rates of *C. pneumoniae* and *M. pneumoniae* than in our study but similar results with respect to *L. Pneumophila* detection(Al-ssum, and Al-Malki, ,2010).

Ina similar study in Japan by Naoyuki *et al.*,(2004) in a total of 208 CAP Cases there were 31(14.9%) cases (13(6.3%) for *C. pneumoniae*, 9(4.3%) for *M. pneumoniae*, 1(0.5%)for dual infection with *C. pneumoniae* and *M. pneumoniae* and 8(3.8%) for *L. pneumophila*) in a sharp disagreement with our result. These may be attributed to antimicrobial resistance due to overuse of antibiotic treatment often with inappropriate antibiotics for atypical pathogens. Delayed or a prolonged treatment, or asymptomatic infection in our patients.

In Egypt, the prevalence of atypical pathogen in CAP was 12/ 400 (3%) by using multiplex PCR. and *Mycoplasma pneumoniae* was determined as the cause of CAP in 4/400 (1%) patients. None were positive for either *C. pneumoniae* or *Legionella pneumophilia* by PCR. (Basha *et al.*, 2019).Similar to our study, these authors indicated no significant difference in clinical presentations of infections.

In our study the high prevalence rate of *L. pneumophila* and mixed infections (mainly due to mixed infection with *L. pneumophila*) could indicate the high abundance of this organism in the environment. On the other hand it is hard to explain why males are more infected then females even in the younger age group. Further studies may be needed to explain this observation. The prevalence of single infection with *Mycoplasma* spp and *C. pneumoniae* were low indicating lower prevalence of these organisms in the environment or otherwise may be related to the transmission modes of these organisms.

Conclusion

Legionella, showed high prevalence while Chlamydia and Mycoplasma showed low prevalence in patients with respiratory infections in Khartoum State and can be detected by using Multiplex PCR. . In addition, our study may indicate that these infections are not appropriately covered by current antibiotic guidelines and lack of proper diagnosis of atypical pathogens.

References

[1] Le Huong P, Hien PT, Lan NT, Binh TQ, Tuan DM, Anh DD. First report on prevalence and risk factors of severe atypical pneumoniae in Vietnamese children aged 1-15 years. BMC Public Health. 2014; 14:1304.

[2] Principi N, Esposito S, Blasi F, Allegra L; Mowgli study group. Role of Mycoplasma pneumoniae and Chlamydia pneumoniae in children with community-acquired lower respiratory tract infections. Clin Infect Dis 2001; 32:1281–1289.

[3] Welti, M., Jaton, K., Altwegg, M., Sahli, R., Wenger, A. and Bille, J. Development of a multiplex realtime quantitative PCR assay to detect *Chlamydia pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* in respiratory tract secretions. *Diagnostic Microbiology and Infectious Disease* 2003; 45, 85-95.

[4] Sung, H., Kang, S. H., Bae, Y. J., Hong, J. T., Chung, Y.B., Lee, S. K. and Song, S.. PCR-based detection of Mycoplasma sp. Journal of Microbiology 2006; 44, 42-49.

[5] Phares, C. R., Wangroongsarb, P., Chantra, S., Paveenkitiporn, W., Tondella, M. L., Benson, R. F., Thacker, W. L., Fields, B. S., Moore, M. R., Fischer, J., Dowell, S. F. and Olsen, S. J. Epidemiology of severe pneumoniae caused by Legionella longbeachae, Mycoplasma pneumoniae, and Chlamydia pneumoniae. Clinical Infectious Diseases 2007; 45, 145-155.

[6] Welti, M., Jaton, K., Altwegg, M., Sahli, R., Wenger, A. and Bille, J. Development of a multiplex realtime quantitative PCR assay to detect Chlamydia pneumoniae, Legionella pneumophila and Mycoplasma pneumoniae in respiratory tract secretions. Diagnostic

[7] Microbiology and Infectious Disease 2003; 45, 85-95.

[8] Elkholy, A., Elkaraksy, H., Fattouh, A., Bazaraa, H., Hegazy, R. and AbdElhalim, M. Acute lower respiratory tract infection due to Chlamydia and Mycoplasma sp. in Egyptian

children under 5 years of age. Journal of Tropical Pediatrics 2009; 55, 195-197.

[9] Reinhard B. Raggam, Eva Leitner, Jörg Berg, Gerhard Mühlbauer, Egon Marth and Harald H. Kessler. Single-run, parallel detection of DNA from three pneumoniae producing bacteria by realtime polymerase chain reaction. Journal of Molecular Diagnostic 2005; 7,133-138.

[10] Matsukura, Tajiri, T., Kato, S., Togashi, A., Masuda, G., Tokunaga, A. and Yamada, N. Diagnostic value of culture, histology and PCR for Helicobacter pylori in the remnant stomach after surgery. Blackwell Publishing Ltd. 2004; 6, 33- 38.

[11] Ho, P.L., Cheng, V.C. & Chu, C.M. Antibiotic resistance in community acquired pneumoniae caused by Streptococcus pneumoniae, methicillinresistant Staphylococcus aureus, and Acinetobacter baumannii. Chest 2009; 136(4): 1119-1127.

[12] Erin A. Mc Donough, Christopher P. Barrozo, Kevin L. Russell, David Metzgar Multiplex PCR for Detection of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis* in Clinical Specimens 2005. *Mol Cell Probes*. 2005; 19(5):314-22.

[13] Al-ssum, R. M.* and Al-Malki, M. A. A Use of multiplex PCR for diagnosis of bacterial infection respiratory mixed 2010 *Mal. J. Microbiol*. Vol 6(1), pp. 1-6.

[14] Schaechter M, Engleberg NC, Eisenstein BI, Medoff G. Mechanisms of Microbial Disease. Third Edition ed. Baltimore: Lippincott Williams and Wilkins, 1999 (Kelly PJ, Ed.

[15] Phares C R, Wangroongsarb P, Chantra S, Paveenkitiporn W, Tondella M L, Benson R F, Thacker W L, Fields B S, Moore M R, Fischer J, Dowell S F and Olsen S J Epidemiology of severe pneumoniae caused by *Legionella longbeachae*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*. *Clinical Infectious Diseases* 2007; 45, 145-155.

[16] Naoyuki M, Atsushi S, Shigeru K, Keizo Y, Akira W, Hiroshi O, Yukumasa K, Toshiharu M. Multiplex PCR for the simultaneous detection of Chlamydia pneumoniae, Mycoplasma pneumoniae and Legionella pneumophila in community-acquired pneumoniae *JResp Med* 2004 98, 542–550.

[17] Noussa R. E, Hala H. S, Hassan A. E, May M. S, Amani A. E. The use of multiplex PCR for the detection of atypical pathogens in Egyptian children with CAP: a high rate of Bordetella pertussis in early infancy *Journal of the Egyptian Public Health Association* (2019) 94:5.(17)

[18] Mustafa O.M, Hamed elnil F.Y. Isolation and Identification of *Legionella pneumophila* from Environmental Water Sources in Khartoum State. *I J A S T Research.* 2013. 56:3:309.

[19] Rabih W M, Kafi S K, Eldowma E Y, Khalid A. The Rate of Detection of Legionella pneumophila IgG Antibodies among Patients with Chest Infection in Major Hospitals in Khartoum State, Sudan. *A J R C* (2014), 2(9): 185-191.

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