Malaysian Journal of Microbiology, Vol 6(1) 2010, pp. 1-6

Use of multiplex PCR for diagnosis of bacterial infection respiratory mixed

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Received 6 March 2009; received in revised form 11 May 2009; accepted 19 May 2009

ABSTRACT

Atypical bacteria grow very slowly in culture or they do not grow at all leading to delays in detection and diagnosis. PCR multiplex was performed on template DNAs extracted from seventy three collected specimens. Thirty seven showed positive indication for the presence of bacterial infection. The incidence of *Mycoplasma pneumoniae*, *Chlamydia pneumonia* and *Legionella pneumophila* as a single infecting agent was 31.5%, 27.5% and 20 % respectively. Dual agent 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%. The etiology of the infection was *M. pneumoniae*, *L. pneumophila* or *C. pneumoniae* as a single etiology or in combination of two or three organisms.

Keywords: Chlamydia pneumonia, Epidemiology, *Legionella pneumophila*, *Mycoplasma pneumonia*e

INTRODUCTION

Acute upper respiratory infections (URI) usually benign, transitory self limited, but it could be serious disease in children and young infants (Phares *et al*., 2007). Infection of the lower respiratory tract, especially pneumonia, can be severe or fatal. Atypical bacteria (*Mycoplasma, Legionella* and *Chlamydia*) are the dominant pathogens; accounting for much higher percentage of lower than upper respiratory tract infections (Welti *et al*., 2003). Lower respiratory tract infections (LRTIs) continue to be important cause of community-acquired pneumonia and therapy is largely empiric because of the inability to determine the causative microorganisms of most patients by the time treatment is initiated (Elkholy *et al*., 2009).

The classical microbiological identification techniques can not provide an efficient means of diagnosis because most of the atypical bacteria grow either slowly or not at all in culture, leading to delays in detection and diagnosis (Sung *et al*., 2006). These problems lead to the emergence of other diagnostic methods, such as L. *pneumophila* antigen, serologic testing by microimmunofluorescence or compliment fixation assays and indirect immuno-enzyme assay (ELISA) which also demonstrated a lack of sensitivity and specificity (Kumar *et al*., 2007; Elkholy *et al*., 2009). Thereafter, nucleic acid amplification based techniques have been developed to diagnose the infections caused by these pathogens (Reinhard *et al*., 2005). Such PCR assays have lessened the importance of culture as means for detecting *M. pneumoniae, L. pneumophila* and *C. pneumoniae;* showing a sensitivity greater than culture (Matsukura *et al*., 2004).

Multiplex PCR assay was developed to detect *M. pneumoniae, L. pneumophila* and *C. pneumonia* (Kate *et al*., 2003). This study has been designed to investigate and evaluate the incidence of single, dual and multiple respiratory bacterial infections using PCR multiplex.

MATERIALS AND METHODS

Materials

A total of 236 clinical specimens (118 males, 118 females) were obtained from King Saud Chest Hospital (Sahari Hospital) in Riyadh city. The sample volume was 7,000 µL to 1,000 µL, types of samples were from pleural fluid (which have been taken in accordance with patients permission). The specimens for immediate treatment were stored at 4 ℃; others at –20 ℃. Qiagen DNA minikit protocol was followed to prepare the reagents. QIAGEN Protease stock solution was stored at 2–8 ℃. A 5.5 mL protease solvent was pipetted into the vial containing lyophilized QIAGEN. AL buffer was stored at room temperature, 15–25 ℃, it was mixed thoroughly by shaking before use. An appropriate amount of ethanol (96–100%) was added to buffer AW1 and AW2 before use and stored at room temperature.

Sample Processing

A 200 µL of each sample was pipetted into a 1.5 mL micro centrifuge tube. A 1000 µL of phosphate buffer saline (PBS) was added and mixed by vortexing for 10 s and centrifuged at 14,000 rpm for 15 s. The supernatant was

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discarded and the pellet was resuspended in 200 µL of PBS and mixed by vortexing for 15 s.

DNA extraction

A 20 µL protease was pipetted into the bottom of a 1.5 mL microcentrifuge tube containing 200 µL of sample and 200 µL AL buffer was added to the sample. This was followed by pulse-vortexing for 15 s. The sample was then incubated at 56 ℃ for 10 min and centrifuged briefly. A 200 µL ethanol (96–100%) was added, vortexed for 15 s. The 1.5 mL microcentrifuge tube was 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. The QIAamp Mini spin column was placed in a clean 2 mL collection tube and the tube containing the filtrate was discarded. A 500 µL AW1 buffer was added, centrifuged at(6000 x *g*) for 1 min., 500 µL AW2 buffer was added, centrifuged at full speed (20,000 x *g*) for 3 min. The QIAamp Mini spin column was placed in a clean 1.5 mL microcentrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was opened, 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. To increase the yield of the eluted DNA, further incubation at room temperature for 5 min and re-elution of the first yield was necessary. The DNA was concentrated with isopropanol and ammonium acetate and washed with 200 µL of 75% ethanol, evaporated by incubating at 65 ℃ for 5 min and dissolved in 10 µL deionized water.

Primers and standard control organism were purchased from Maxim Biotech. Inc. USA. Primers sequences are as follow:

5'-GTTGTTCATGAAGGCCTACT-3' forward
5'-TGCATAACCTACGGTGTGTT-3' reverse
5′-ACCGATGCCACATCATT-3' forward
5'-ACGACCAGTGTATTCGACAG-3' reverse
5'-TCAATCTGGCGTGGATCTCT-3' forward
5'-GTCACTGGTTAAACGGACTA-3' reverse

Multiplex PCR profile

Reaction volume

A 25.0 µL, 2x PCR buffer, 5.0 µL 10x primers, 0.5 µL Taq polymerase (5 U/µL), 5.0 µL DNA, 14.5 µL H_2O, total volume 50 µL.

PCR Program

94 ℃, 2 min for 1 cycle, 96 ℃, 1.30 min for 2 cycles, 63 ℃, 4 min for 2 cycle, (94 ℃, 1.30 min, 62 ℃, 2.30 min for 35 cycles), 70 ℃, 10 min for 1 cycle and 4 ℃ for cooling. Agarose gel electrophoresis was used to visualize multiplex PCR DNA product and photographed under UV.

RESULTS AND DISCUSSION

Thirty seven out of seventy three specimens showed positive indication for the presence of bacterial infection. The incidence of *M. pneumoniae*, *C. pneumonia*, *L. pneumophila* as a single infecting agent was 31.5%, 27.5% and 20% respectively. Dual agent 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%. The etiology of the infection was *M. pneumoniae*, *L. pneumophila* and *C. pneumoniae* as a single etiology or in combination of two or three organisms was positive for HIV infection. The multiplex PCR results are presented in Figure 1 and detailed in Table 1. Figure 2 presents the percentage of patients infected by single and multiple agents. *M. pneumoniae* is one of the causative agents of atypical community-acquired pneumoniae (Salvatore *et al*., 2009). However, it is associated with the aggregate number of chronic infectious burden of atypical respiratory pathogens such as *C. pneumonia*, *M. pneumoniae* and *L. pneumophila*.

Multiple studies have suggested an association between *C. pneumoniae* and *M. pneumoniae* infection and cardiovascular disease (Ngeh *et al*., 2005). Respiratory tract infections account more than 10% of all office visit to the primary care physician and restrict the activities of children in certain age category (5-17 years) due to acute condition (Phares *et al*., 2007). On the other side community acquired pneumonia is the sixth leading cause of death and 25% of patients are requiring hospitalization (Nawalutfiyya *et al*., 2006). Mortality among patients with CAP has risen due in part to an increasing elderly population (Liu *et al*., 2004). A typical pneumonia caused by *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* as a single agent or mixed infection has been detected in 50.68% of all selected and studied specimens. It is an alarming situation which should be taken seriously by health care system management and the search for tools and measures to reduce such high percentage of infection is a necessity. The question which should be raised concerning this observation, are Saud's citizen more susceptible to pneumonia infection or it is the inadequate management within the hospital or even both. Unfortunately we do not have the answer without investigating each part of the question.

However, we care for the patients because the treatments for *M. pneumoniae, C. pneumoniae* and *L. pneumophila* as a single infecting agent may be achievable but, when the disease is a result of mixed infection it is hard to treat in the absence of knowledge about the etiological agent. In mixed infection treatments of a single agent may be inhibited and decrease the number and activity of the organism leading to the improvement of the patient. By contrast, due to inhibition of one organism the other may thrive causing the onset of new suffering period and so on. Death has been observed among hospitalized patients but our conclusion was hindered by an underling disease e.g. TB and HIV.

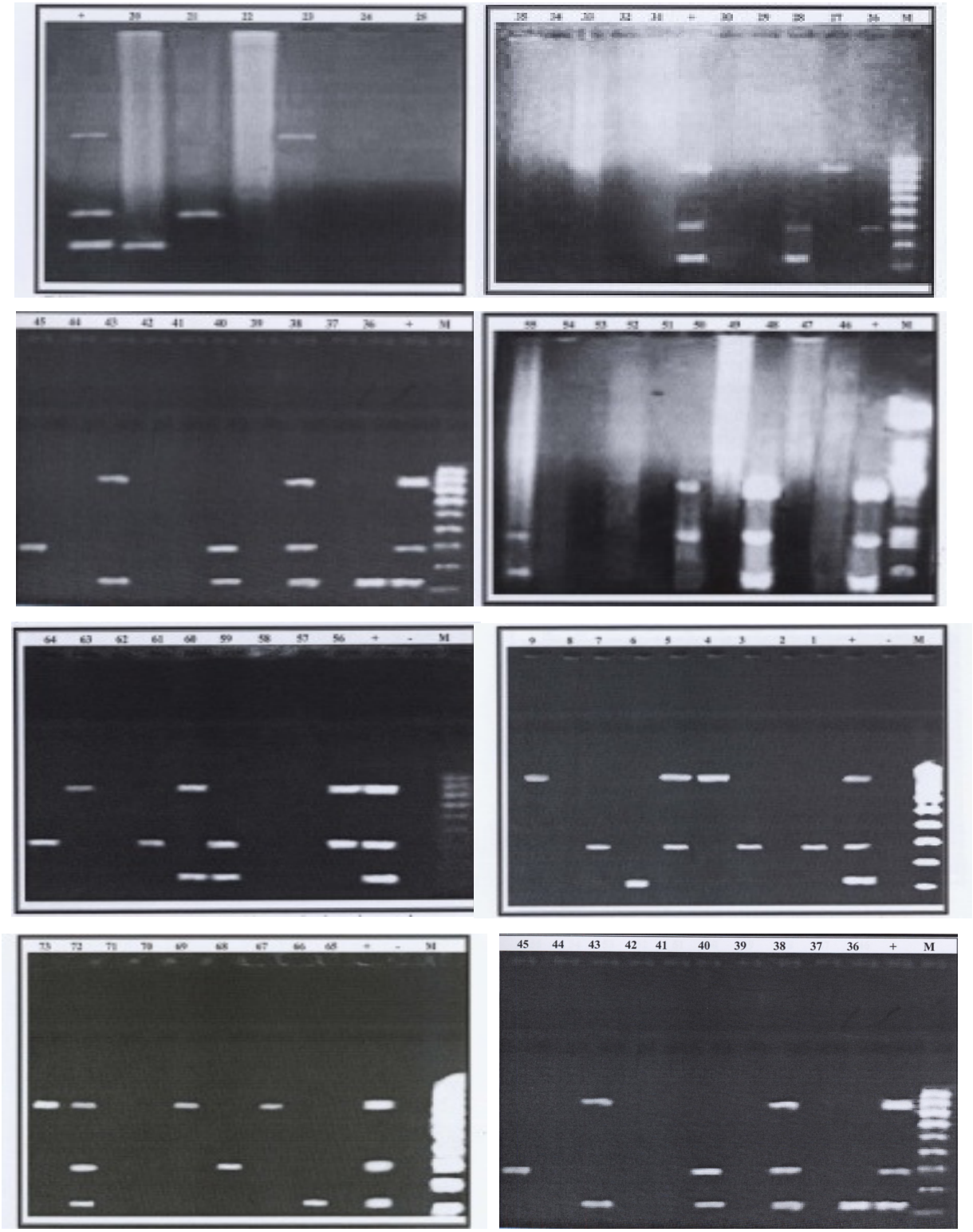

Figure 1: Multiplex PCR results of patients showing single, double and triple infection. Lane M: Marker; Lane (+): Positive control; Lane 36: *Legionella*; Lane 38: *Mycoplasma*, *Legionella* and *Chlamydia*; Lane 40: *Mycoplasma* and *Legionella*; Lane 43: *Legionella* and *Chlamydia*; Lane 45: *Mycoplasma*

Table 1: Interpretation of multiplex PCR results

Patients	Nationality	Prediagnosing	*Mycoplasma pneumoniae*	*Legionalla pneumophila*	*Chlamydia pneumonia*
1	Saudi	Resp	+	-	-
2	Saudi	TB	-	-	-
3	Saudi	Resp	+	-	-
4	Saudi	Resp	-	-	-
5	Saudi	TB	+	-	+
6	Saudi	Resp	-	+	-
7	Saudi	Resp	+	-	-
8	Saudi	Resp	-	-	-
9	Saudi	Resp	-	-	+
10	Saudi	Reasp	+	+	-
11	Saudi	Reasp	-	-	-
12	Saudi	TB	-	-	+
13	Saudi	Reasp	+	-	-
14	Saudi	TB	+	-	+
15	Saudi	Reasp	-	-	-
16	Saudi	Reasp	+	+	-
17	Saudi	TB	-	-	-
18	Saudi	Reasp	-	-	-
19	Saudi	Reasp	-	-	+
20	Saudi	TB	-	+	-
21	Saudi	TB	+	-	-
22	Saudi	Reasp	-	-	-
23	Saudi	Reasp	-	-	+
24	Saudi	TB	-	-	-
25	Saudi	Reasp	-	-	-
26	Saudi	Reasp	+	-	-
27	Saudi	TB	-	-	+
28	Saudi	Reasp	+	+	-
29	Saudi	Reasp	-	-	-
30	Saudi	TB	-	-	-
31	Saudi	Reasp	-	-	-
32	Saudi	Reasp	-	-	-
33	Saudi	Reasp	-	-	+
34	Saudi	Reasp	-	-	-
35	Saudi	Reasp	-	-	-
36	Saudi	Reasp	-	+	-
37	Saudi	TB	-	-	-
38	Saudi	HIV+ Resp	+	+	+
39	Saudi	Reasp	-	-	-
40	Saudi	TB	+	+	-
41	Saudi	Reasp	-	-	-
42	Saudi	Reasp	-	-	-
43	Saudi	Reasp	-	+	+
44	Saudi	TB	-	-	-
45	Saudi	Reasp	+	-	-
46	Saudi	TB	-	-	-
47	Saudi	Reasp	-	-	-
48	Saudi	TB	+	+	+
49	Saudi	TB	-	-	-
50	Saudi	Reasp	+	-	+
51	Saudi	Reasp	-	-	-
52	Saudi	Reasp	-	-	-
53	Saudi	Reasp	-	-	-
54	Saudi	Reasp	-	-	-
55	Saudi	Reasp	+	+	-
56	Saudi	Reasp	+	-	+
57	Saudi	Reasp	-	-	-
58	Saudi	Reasp	-	-	-
59	Saudi	HIV	+	+	-
60	Saudi	TB	-	+	+

Cont.

61	Saudi	Reasp	+	-	-
62	Saudi	TB	-	-	-
63	Saudi	Reasp	-	-	+
64	Saudi	Reasp	+	-	-
65	Saudi	Reasp	-	-	-
66	Saudi	Reasp	-	+	-
67	Saudi	Reasp	-	-	+
68	Saudi	TB	+	-	-
69	Saudi	TB	-	-	+
70	Saudi	Reasp	-	-	-
71	Saudi	TB	-	-	-
72	Saudi	Reasp	+	+	+
73	Saudi	Reasp	-	-	+

However, we conclude that the use of such multiplex PCR systems would improve the management of adult patients with *M. pneumoniae, C. pneumonia* and *L. pneumophila* related to nosocomial respiratory bacterial infections. Such molecular assay would be also valuable particularly with regard to disease control. Moreover, the single, dual and triple etiology of genetically distinct organisms indicates the possibility of repeated respiratory infections.

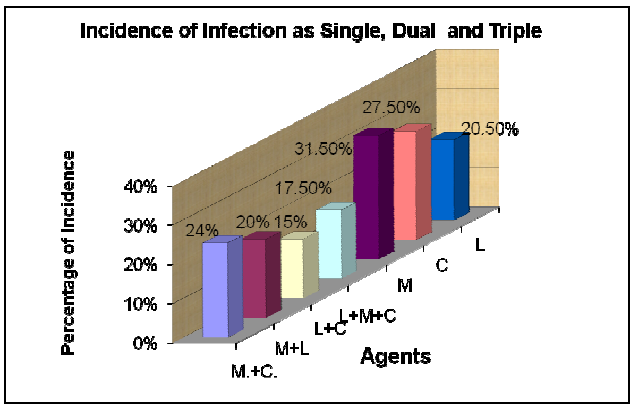

Figure 2: Percentage of patients infected by single and multiple agent

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