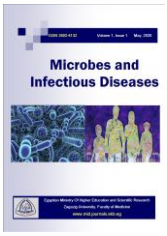
Microbes and Infectious Diseases 2024; Article-In-Press, **DOI:** 10.21608/mid.2024.249973.1670



Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Indirect immunofluorescent assay for rapid detection of atypical respiratory tract bacteria among septic patients

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ARTICLE INFO

Article history:
Received 20 November 2023
Received in revised form 28 December 2023
Accepted 8 January 2024

Keywords:
Lower respiratory tract
Indirect immunofluorescent assay
Legionella
Coxiella
Mycoplasma
Chlamydophila

ABSTRACT

Background: Atypical bacteria are unculturable and unique infectious agents that can infect the lower respiratory tract (LRT), cause atypical pneumonia, and may develop bacteremia. The purpose of the study was to identify if the elderly hospitalized patients who reside in intensive care units had atypical pneumonia. **Material and methods:** A total of 40 serum samples from septic patients with an age mean of 68.6 ± SD 8.5 in the hospitals of the Babylon Health Directorate were collected. All serum samples were tested using an indirect immunofluorescent assay (IFA) to detect IgM and IgG antibodies specific for *Legionella pneumophila*, *Coxiella burnetii*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *and Chlamydophila psittaci*. **Results:** The results showed that these bacteria were present as single and/or multiple pathogens in 25 (62.5%), 2 (5%), 10 (25%), 25 (62.5), 4 (10%) of 40 samples, respectively. **Conclusions:** This investigation proves the presence of specific antibodies against various atypical pulmonary pathogens among septic patients with unidentified etiological agents.

Introduction

Sepsis is a spectrum of diseases and systemic immune response to injury by pathogens [1]. However, infectious illnesses are the most common cause of mortality throughout the world [2]. The presence of pathogens such as bacteria in the bloodstream for an extended period can cause a catastrophic body-wide response, particularly in the elderly [3,4]. The current investigation is focused on pulmonary bacteria, which infect the lower respiratory tract and cause community and/or hospital acquired pneumonia and represent one of the most important etiologic agents of sepsis. Some of these bacteria are so-called atypical bacteria that can cause a particular type of pneumonia (atypical pneumonia) and can synergize with other pathogens such as cold viruses, resulting in coinfection [5].

Lower respiratory tract infections lead to more deaths than any other form of infectious disease, and the increase in the number of deaths may be due to the increase in population and aging [6]. The main reason for death is related to delayed identification of the causative pathogens. Failure of early detection of the pathogenic agent may result in adverse effects. So, fatal sickness must be diagnosed as soon as possible so that it can be treated [7,8].

Even though culture methods represent the gold standard diagnostic test [9], this method is insufficiently sensitive, especially in the presence of atypical and fastidious bacteria. A serological test is typically used to diagnose negative blood culture

DOI: 10.21608/MID.2024.249973.1670
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diseases caused by fastidious bacteria. One of the most available serological methods are those that detect multiple pathogens per test and require only a single dilution, thus helping rapid diagnosis [10]. Atypical bacteria such as *Legionella pneumophila*, *Coxiella burnetii*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *and Chlamydophila psittaci are* unculturable and do not color with Gram and Ziehl-Neelsen stain [11]. While it is impossible to isolate these bacteria and extract them from clinical samples via cultivation, the standard method for detection depends on the use of polymerase chain reaction (PCR) and/or immunofluorescent assay [12].

An indirect immunofluorescent assay (IFA) was used in the present study to detect the main five microorganisms that cause atypical pneumonia in hospitalized patients with unknown etiological agents. This method used primary unconjugated and secondary fluorophore-conjugated antibodies directed against antigen-specific antibody.

Material and methods

Patient choice

Case records of patients who had a laboratory checkup were reviewed. All of them reside in the intensive care units and had positive c-reactive protein, abnormal white blood cell counts, and a negative result of blood culture.

Ethical approval

The first stage must be acceptance, in which a patient consents to participate in research and permits the collection of information and their medical history. The Babylon Health Directorate approved of the ethical stance. A professional safety protocol was applied throughout sample handling. The approval number and date of this study was 28796 on 2 January 2022.

Sample collection

During the study period from January to June 2022, a total of 40 septic patients between the age of 56 to 90 were included. They were all admitted to the intensive care units (ICU) of Hilla hospitals. A volume of 2 mL of blood samples were collected from all participants in the early morning.

Indirect immunofluorescent assay protocol

All serum samples were tested for IgG and IgM antibodies against the main bacterial agents that cause atypical pneumonia: *Legionella pneumophila*, *Coxiella burnetii*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Chlamydophila psittaci* using the PNEUMOBACT IFA kit supplied by (Vircell, Spain).

The procedure was performed in accordance with the manufacturer's instructions. The IFA procedure is based on the response of antibodies in the sample (serum or plasma) when tested with an antigen that has been adsorbed on the slide surface. The specific antibodies in the samples reacted with the antigen, whereas any non-specific immunoglobulins that are not bound to the antigen were removed during the washing step. The antigen-antibody complexes then interacted with the fluorescein-labelled anti-human globulin in the next step and were examined with an immunofluorescence microscope.

Results

The findings revealed that each of anti-*Legionella pneumophila* and anti-*Chlamydia pneumoniae* antibodies was present in 25 (62.5%) of the 40 samples, whereas anti-*Mycoplasma pneumoniae* was detected in 10 (25%) of the 40 samples. All these pathogens belong to the common nonzoonotic atypical bacteria. Regarding zoonotic atypical pathogens, when the serum samples were examined, anti-*Coxiella burnetii* and anti-*Chlamydia psittaci* antibodies were found in 2 (5%) and 4 (10%) of the 40 cases, respectively. The numbers and percentages of each anti-bacterial IgG and IgM were documented in **Table (1)**.

Multiple infections were present (each test gave positive reactions to different specific antibodies that belonged to more than one microorganism). Coinfection was detected in 24 (60%) of the 40 patients; 11 (27.5%) of them had *L. pneumophila* and *C. pneumoniae*; 6 (15%) had *L. pneumophila*, *C. pneumoniae* and *M. pneumoniae*; 3 (7.5%) had *C. pneumoniae* and *M. pneumoniae*; 2 (5%) had *C. pneumoniae* and *C. psittaci*; 1(2.5%) had *L. pneumophila* and *C. burnetii*; 1(2.5%) had *L. pneumophila* and *C. psittaci* **(Table 2)**.

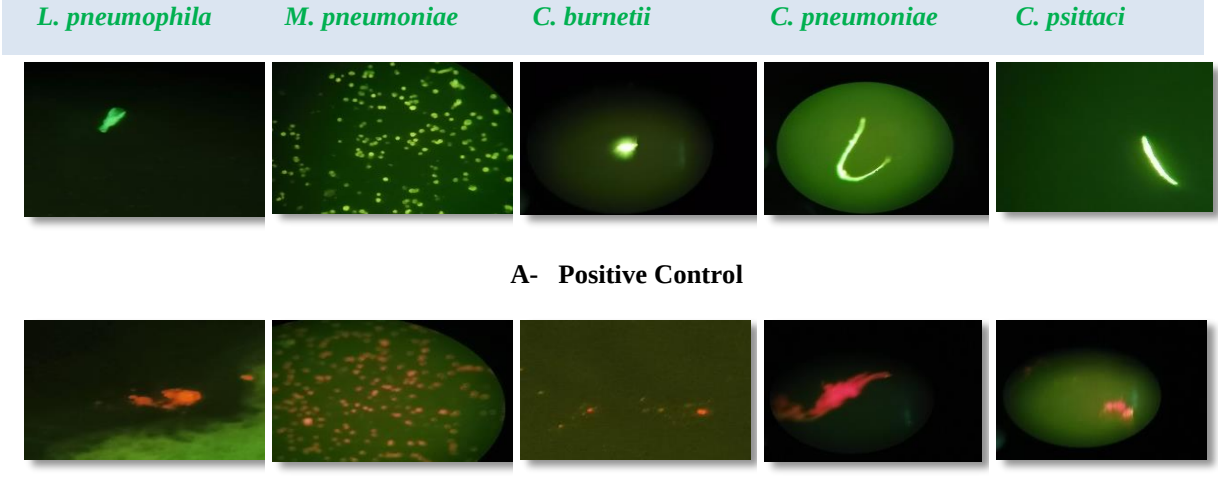
Table 1. The number and percentage of each human IgM and IgG antibody detected out of 40 serum samples examined by the IFA method.

Bacterial Antigen	**Positive results %**			**Total Positive%**
	Human IgM	Human IgG	Both IgM and IgG	
L. pneumophila	8 (20%)	3 (7.5%)	14 (35%)	25 (62.5%)
C. pneumoniae	6 (15%)	7 (17.5%)	12 (30%)	25 (62.5%)
M. pneumoniae	1 (2.5%)	2 (5%)	7 (17.5%)	10 (25%)
C. psittaci	1 (2.5%)	2 (5%)	1 (2.5%)	4 (10%)
C. burnetii	0 (0%)	0 (0%)	2 (5%)	2 (5%)
Total number of positive =36 of 40 serum samples				

Table 2. The number of single and multiple pathogens that were present in each sample.

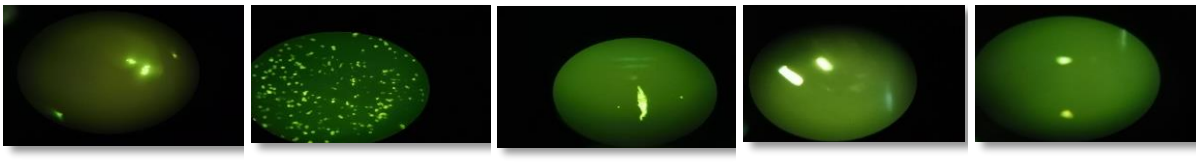
Single Pathogen	**NO.**
L. pneumophila	6
M. pneumoniae	1
C. burnetii	1
C. pneumoniae	3
C. psittaci	1
Multiple pathogens	**NO.**
L. pneumophila, C. pneumoniae	11
L. pneumophila, M. pneumoniae, C. pneumoniae	6
L. pneumophila, C. burnetii	1
C. pneumoniae, C. psittaci	2
L. pneumophila, C. psittaci	1
M. pneumoniae, C. pneumoniae	3
Total number of positive	**36**

Figure 1. IFA images (the green apple color refers to positive results while red indicates to negative results).

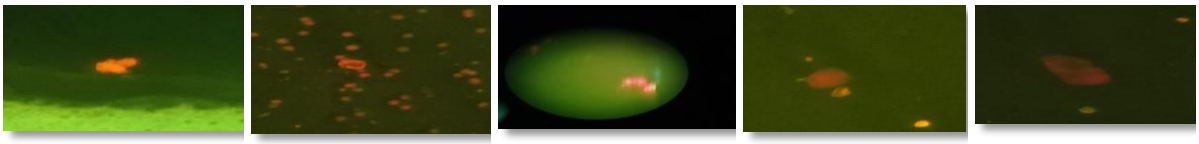


A- Positive Control

B- Negative Control



C-Positive Results



D-Negative Results

Discussion

Respiratory tract infections are the subject of recent advancements in medical field research because of their wide distribution and the high rates of morbidity and mortality reported all over the world [13]. The main atypical bacteria that infect LRT are classified into zoonotic and non-zoonotic pathogens. *Chlamydia psittaci* and *Coxiella burnetii* are transmitted by animals, whereas *Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae* are the most prevalent and non-zoonotic in origin [14].

Each type of these bacteria has the tendency to extrapulmonary involvement and is more probable to result in systemic disease. However, it is difficult to cultivate and diagnose these microorganisms [15]. For these reasons, a prompt clinical diagnosis is critical for raising the index of suspicion and commencing appropriate therapy. The present methodology for the laboratory diagnosis of sever LRTIs and other systemic illnesses depends on several tests including traditional culture, IFA, and other rapid serological tests [16,17,18].

In the current study, the results of the IFA assay demonstrate differences among all tested cases regarding the spectrum of disease. Some cases gave only positive results for IgM antibodies indicating the presence of recent infection or acute phase of the disease [19]. However, other cases gave positive results for IgG indicating past exposure or reinfection [20]. The remaining infected patients gave positive results for both IgM and IgG indicating the progression of illness episodes that may become severe. Overall, the findings revealed that *Legionella pneumophila* was present in 25 (62.5%) of 40 serum samples. It represented the largest percentage among other detected atypical bacteria. *Legionella pneumophila* is a small Gram-negative bacillus normally present ubiquitously, especially in water sources such as showerhead and taps [21], transmitted via inhalation of contaminated aerosols and causes atypical pneumonia (Legionnaires) that can be community-acquired or hospital-acquired. *Legionella* is the most significant atypical bacteria that can lead to severe community-acquired pneumonia in hospitalized patients [22].

Prior research has shown that *L. pneumophila* may breach the pulmonary blood vessels as a potential initiating event in bacteremia during the systemic spread of the bacteria through the bloodstream [23]. In 2002, an international survey performed by **Yu *et al.*** recognized community-acquired legionellosis in 508 patients; 91.5% of the isolates belonged to *Legionella pneumophila* and serogroup 1 was the predominant 84.2% [24]. The IFA is advised as the reference approach for the diagnosis of *Legionella pneumophila*-associated respiratory tract infection, with a sensitivity of between 75% and 80% and a specificity of greater than 99% when the *Legionella pneumophila* serogroup 1 antigen is used [25].

Of the 40 serum samples, *Chlamydia pneumoniae* was present in 25 (62.5%), the second-highest percentage among other detected atypical bacteria. *Chlamydia pneumoniae* is an obligate intracellular, Gram-negative bacterium present in two developmental forms: elementary and reticulate bodies [26]. As with *L. pneumophila*, transmission occurs through inhalation of contaminated droplets, and its pathogenicity involves extrapulmonary organs. Depending on several previous studies, *C. pneumoniae* is recognized as the most common non-viral intracellular human respiratory pathogen. It

accounts for 6-20% of community-acquired pneumonia (CAP). The spectrum of its infectivity varies from mild to severe especially in elderly and immunocompromised patients [27].

Mycoplasma pneumoniae is another bacterial pathogen that lacks a cell wall and belongs to the prevalent nonzoonotic atypical infection. It was detected by the IFA approach in 10 (25%) of the 40 serum samples. According to previous study, *M. pneumoniae* causes approximately 10 to 30 percent of all cases of CAP, particularly among immunocompromised patients [28].

Regarding zoonotic atypical pathogens, *Coxiella burnetii* and *Chlamydia psittaci* were found in 2 (5%) and 4 (10%) of the 40 cases, respectively. Zoonotic atypical pathogens are less common than nonzoonotic due to their connection to environmental risk factors and the exposure to specific vectors [29].

It is noteworthy that this investigation showed that multiple infections were present when the serum samples were tested as shown in **Table (2)**. The incidence of coinfection is higher in debilitated patients, particularly those reside in the intensive care unit or respiratory care unit. This is true because more than three quarters of patients' samples were collected from hospitalized patients who reside in those units. A retrospective study conducted by **De Francesco *et al.*** targeted 721 hospitalized patients; the demographic distribution of their ages was like that of the participants in the current study (median ages ~65 years). This study showed out of 443 patients suffering from SARS-CoV-2, 242 had an antibody against *Mycoplasma* and *Chlamydia* [30]. Moreover, **Goodarzi *et al.*** detected coinfection in 7 patients; 6 of them were infected by *M. pneumoniae* and *L. pneumophila*, and only one was infected by *L. pneumophila* and *C. pneumoniae* [31].

Conclusion

This investigation proved the presence of specific antibodies against various atypical pathogens among elderly patients who have systemic infections from unidentified etiological agents. The IFA test is a rapid and highly sensitive technique that must be used along with conventional blood culture and other routine diagnostic methods to accurately identify etiological agents. Finally, there is an overall idea relates to constantly updating the methods of diagnosis, which may lower the death rate and help patients to recover quickly.

Funding

This research received no external funding

Conflicts of Interest

The authors declare no conflict of interest.

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