

# Legionella Pneumophila Infections among Egyptian Patients: Updated Diagnostic Tests and Evaluation

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**Background and study aims:** Legionella pneumophila is a gram-negative bacterium, which is implicated in causing Legionnaires' disease and Pontiac fever. This study aims at evaluating the different updated diagnostic tests of L. pneumophila infections among pneumonic patients through the detection of L. pneumophila urinary antigen testing (UAT), and quantitative Real Time PCR (RT PCR) in comparison to the gold standard test and to detect the prevalence of pneumonia caused by L. pneumophila.

**Patients and Method:** One hundred patients with features suggestive of pneumonia and confirmed by imaging were enrolled in this study. Serum, urine and lower respiratory specimens were obtained from patients admitted to Chest and Tropical Medicine Departments, Zagazig University Hospitals. Patients were subjected to urinary antigen test, lower respiratory specimens' cultures and

q Real Time (RT) PCR for detection of L. pneumophila mip gene.

**Results:** Prevalence of L. pneumophila in Zagazig university hospitals was 17% detected by L. pneumophila urinary antigen testing (LPUAT), 15% of samples were positive by q RT PCR based mip gene and 12 % positive by respiratory sample cultures. In comparison to respiratory sample culture, the gold standard, q RT PCR evaluation showed sensitivity 83.3%, specificity 94.3%, positive predictive value 66.7%, Negative predictive value 97.6% and accuracy of 93.0%. **Conclusion:** Molecular assays and UAT are promising methods that can be used in the rapid and early diagnosis of Legionella infections but they seem not to be sufficient to replace microbial cultures which must associate these techniques.

## INTRODUCTION

Legionella pneumophila are Gram-negative bacilli and natural aquatic organisms. Infection is secondary to inhalation of contaminated aerosols produced by cooling towers, air conditioners, and showers [1]. Infection leads to the development of Legionnaires' disease (LD) or a flu-like illness called Pontiac fever. Legionnaires' disease represents 2–15% of all cases of community-acquired pneumonia (CAP) in Europe and North America [2]. The community acquired Legionella pneumonia should be treated empirically once suspected, as delayed treatment could lead to severe

pneumonia and associated with poor prognosis [3].

People who are living in warm and rainy areas are more likely to develop Legionella pneumonia due to frequent use of air-conditioning and air-circulating systems [4]. Predisposing conditions for Legionella infection include ageing, male gender, low immunity states, chronic lung disease, and alcohol abuse, along with smoking [3].

The diagnosis of Legionella pneumonia is based on the presence of chest symptoms and fever, together with gut symptoms like abdominal pain and diarrhea. The usual laboratory methods for the diagnosis of LD include culture, urinary antigen testing (UAT), and serological tests. The urinary antigen test is the most popular test in the diagnosis of LD, as it is a rapid, simple and cheap test. However, it has low sensitivity especially in mild to moderate disease activity [5].

The molecular diagnosis of Legionella pneumonia has been commonly used to diagnose LD using Real time PCR (RT PCR) which is based on the amplification of specific DNA sequence that responsible for the entry of legionella inside the macrophages. This technique has the advantages of early diagnosis and higher sensitivity than the culture methods [6].

The aim of this work is to evaluate the performance of different diagnostic tests of Legionella pneumonia infection including q Real Time PCR (RT PCR) and urinary antigen testing (LPUAT) in terms of sensitivity and specificity compared to the gold standard. Additionally, it aimed to determine the prevalence of Legionnaires' disease in pneumonic patients in Zagazig University Hospitals.

## PATIENTS AND METHODS

This cross sectional study was conducted in the Tropical Medicine and the Chest Departments, in collaboration with the Clinical Pathology Department, Zagazig University Hospitals, during the period between March 2022 and March 2023. It included 100 patients, who had pneumonia and confirmed by chest radiography. The inclusion criteria included patients who are  $\geq 18$  years old, and have clinical features suggestive of pneumonia, which was confirmed by chest radiography. In addition, lower respiratory specimens; sputum or broncho-alveolar lavage (BAL) was included. The exclusion criteria included difficult accessibility of lower respiratory secretions, patients with atypical pneumonia and patients, who are less than 18 years old.

### Method:

#### Urine Antigen Test (UAT) for legionella:

Urine samples were collected, concentrated, and examined for the detection of the Legionella

Urinary Antigen using Enzyme Immunoassay Test or Biotest Legionella urine antigen EIA (Biotest AG, Dreieich, Germany) [2].

#### Real-time (PCR) targeting the mip gene:

Serum samples were used for extraction of the legionella DNA using the (DNA- Sorb-B extraction kit) (Sacace TM, Italy). Real-time PCR targeting the mip gene of the Legionella pneumophila was done using (LightCycler® FastStart DNA Master HybProbes). The sequences of the primers were as follows: forward primer (LpmipFp), 5-GCAATGTCAACAGCAA 3; reverse primer (LpmipRp), 5-CATAGCGTCTTGCATG 3. The test was performed according to the manufacturer's instructions using LightCycler 2.0 carousel-based (Roche Diagnostics, Mannheim, Germany) platform [7].

#### Lower respiratory samples (sputum /Broncho-alveolar lavage) culture:

Ninety-six sputum and four broncho-alveolar lavage (BAL) samples were used for the culture on Buffered charcoal-yeast extract agar (BCYE) agar plates with antibiotics (OXOID, Basingstoke, UK) using the standard technique [8]. Aerobic incubation of the plates was done at a temperature of 35-37 for about 10 days. Inspection for growth of the colonies of legionella was done every other day using SLIDEX® LEGIONELLA KIT (50 TESTS) SKU Number; 73120 (Biomereux Marcy l Etoile, France). The kit utilize latex particles coated with antibodies. The Legionella antigens grown in the culture bound to these antibodies coating latex particles are causing visible agglutination in as little as 30 seconds or up to a few minutes [2].

#### Statistical Analysis

The data were analyzed using SPSS version 21.0 Software and Microsoft Excel 2013. Numerical data were described as mean  $\pm$  standard deviation or median. The concordance between tests was evaluated using the Kappa test ( $K < 0.20 =$  "poor";  $0.20-0.40 =$  "fair";  $0.40-0.60 =$  "moderate";  $0.60-0.80 =$  "good";  $0.80-1.00 =$  "very good"). The specificity, sensitivity and the positive and negative predictive values (PPV and NPV, respectively) and 95% confidence intervals (CI) for all methods were calculated considering the Culture of respiratory specimen as a reference method (Gold standard). In addition, the concordance between all methods was also calculated.

## RESULTS

### Demographic data of the studied group:

This study has involved 100 patients with manifestation of pneumonia, 61 (61%) patients were males, and 39 (39%) were females; female to male ratio was 1:1.56. the mean age was 57 years old. the younger age was 18years old while the older was 84 years old.

### Evaluation of real time PCR (q RT PCR):

The study results have revealed that, out of 100 samples from pneumonic patients confirmed by chest radiography (CXR), there were 12 (12%) positive respiratory samples cultures. Additionally, 15 (15%) serum samples (by q RT PCR) were found to be positive. However, there were two samples positive by respiratory samples culture were negative by the PCR, while, there were five samples that were positive by PCR but negative by the culture.

Sensitivity, specificity, predictive values of positive and negative and accuracy were measured in comparison to the respiratory sample cultures (The gold standard for diagnosis of Legionella infection). Sensitivity was 83.3%, Specificity 94.3%, Positive predictive value 66.6%, Negative predictive value 97.6% and accuracy 93.0%. Correlation with the respiratory sample cultures showed a high degree of agreement, which was substantial agreement between the two methods (Kappa agreement  $0.70 \pm 0.008$   $p < 0.001$ ) (Table 1 & Table 3).

### Evaluation of Urinary antigen test (UAT) for legionella:

The results showed that out of 100 samples, 17 (17%) were found to be positive by the UAT. The 12 samples that were positive by respiratory sample culture were also positive by UAT. Moreover, 5 new samples were positive by the UAT and negative by the culture.

Sensitivity, specificity, negative and positive predictive values and accuracy compared with the respiratory sample culture; Sensitivity of 100%, specificity of 94.3%, positive predictive value of 70.6%, negative predictive value of 100% and accuracy of 95%. A 100% for both sensitivity and negative predictive value makes the test as good negative and can be used to rule out of infections. Correlation with the respiratory sample cultures showed a high substantial degree of agreement between the two methods (Kappa agreement  $0.79 \pm 0.009$ ,  $p$  value  $< 0.001$ ) (Table 2 & Table 3)

### The turnaround times:

The lower respiratory specimen`s cultures, the real time PCR and the urinary antigen testing for the Legionella were compared. The Urinary antigen detection showed the shortest turnaround time, which was less than a one hour, followed by the molecular technique, which was less than 4 hours. However, the Lower respiratory specimen`s culture had the longest turnaround time, which was from 3 to 7 days.

**Table 1: Relation between q RT PCR and respiratory sample culture**

		Respiratory sample culture					
		Negative		Positive		Total	
		<i>n.</i>	%	<i>n.</i>	%	<i>n.</i>	%
PCR	Negative	83	83%	2	2%	85	85%
	Positive	5	5%	10	10%	15	15%
	Total	88	88%	12	12%	100	100%

PCR: Polymerase Chain Reaction

**Table 2: Relation between urinary antigen test (UAT) and respiratory sample culture**

		Respiratory sample culture					
		Negative		Positive		Total	
		<i>n.</i>	%	<i>n.</i>	%	<i>n.</i>	%
UAT	Negative	83	83%	0.0	0.0%	83	83%
	Positive	5	5%	12	12%	17	15%
	Total	88	88%	12	12%	100	100%

**Table 3: Accuracy of the respiratory sample culture versus PCR and UAT**

	Respiratory sample culture						
	Specificity	Sensitivity	Positive predictive value	Negative predictive value	Accuracy	Kappa coefficient	P
PCR	94.3%	83.3%	66.6%	97.6%	93%	0.70±0.008	<0.001
UAT	94.3%	100%	70.6%	100%	95%	0.79±0.009	<0.001

## DISCUSSION

In this study, the prevalence of Legionnaires' disease (LD) in Zagazig University Hospitals by using the urinary antigen testing, the molecular technique (q RT PCR mip gene) and the culture of the lower respiratory specimens was 17%, 15% and 12% respectively. It is reported that *Legionella Pneumophila* comprises 2-9% of the cases of community-acquired pneumonia (CAP) [9-13]. The mean incidence of Legionella infection in CAP globally was as follow; Africa 1.6% , Eastern Mediterranean 9.7%, Europe 4.5%, South-East Asian 4.3%, USA 1.5% and Western Pacific 4.5% [14-16]. The large heterogeneity in the incidence estimates for Legionella infections is attributed to many factors including variable definitions of CAP, patients' characteristics, diagnostic methods, and criteria used for diagnosis [15].

The current gold standard test used for the diagnosis of LD is the lower respiratory specimen culture although its use remains challenging, as the culture systems have some limitations, including delay of the results, sample non-accessibility, and low sensitivity, especially

after initiation of antibiotics [17]. To hasten the diagnosis and improve the sensitivity, two diagnostic tools were evaluated, q RT PCR mip gene and urinary antigen test (UAT).

In the present study, out of 100 samples taken from patients with confirmed pneumonia, *Legionella pneumophila* was detected in 12% of the lower respiratory specimen cultures which was in the positivity range of other studies; **Ricci et al.** found that culture positivity rate was 9.9% [18]. Moreover, **Cloud et al.** found a culture positivity rate of 14.6% [19]. On the other hand, several studies showed lower positivity rates 2.9% by **Kese et al.** [2], 5% by **Nageeb et al.** [20] and 2.8% by **Peci et al.** [21]. These differences in positivity of lower respiratory specimen culture results would be attributed to differences in the disease endemicity in different localities and exposure to antimicrobials.

The current study showed that Legionella was detected in 15% of the total samples by molecular technique (q RT PCR mip gene). This improvement in Legionella detection by using PCR goes in accordance with different studies. **Recci et al.** reported that PCR was positive in 53 samples (15%) out of 354 samples, while

respiratory specimen culture was positive only in 35 samples (10%) [18]. While *Kese et al.* reported that out of 3038 samples, PCR detected 128 (4.2%) positive samples, while respiratory specimen culture detected only 88 (3%) positive samples [2].

It was noted that, out of the 15 positive samples by the PCR, five samples were tested negative for the respiratory samples' cultures. This could be interpreted by the detection of non-viable dead or non-cultivable legionella by the PCR [22], as well as contamination of the PCR reagents with the microbial DNA, which will result in false-positive results [23]. Additionally, two of the 12 culture-positive samples, the PCR results were negative. This may be due to inhibition of the PCR reaction, sequence variability underlying the primers and probes, or the presence of Legionella species in quantities less than the limit of detection of the assay [24]. Therefore, a negative PCR result indicates the absence of detectable Legionella's DNA in the specimen but does not rule-out the infection. The presence of two cases, which were diagnosed only by the culture and missed by the molecular technique and also 5 cases with positive PCR and negative culture results makes the interpretation of the PCR should be done cautiously and hand-in-hand with culture which must associates this technique. For evaluating the PCR assay compared to culture, the results showed a sensitivity of 83.3%, a specificity of 94.3%, a positive predictive value of 66.6%, a negative predictive value of 97.6% and an accuracy of 93.0% (Table 3). Moreover, the results obtained by the molecular technique correlated well with those obtained by culture (Kappa co-efficient  $0.70 \pm 0.008$   $p < 0.001$ ). (Table 3). Good concordance with these results was found in other studies using conventional or automated PCR techniques. *Ricci et al.*, showed that, overall concordance between the culture and the PCR was significant ( $k = 0.75$ ;  $p < 0.0001$ ). However, the sensitivity was 63.6%, which was lower than our study, while the specificity was 100%, along with the PPV and the NPV were 100% and 93.7%, respectively [18]. *Mérault et al.* showed that, the PCR and culture results were concordant for 182 out of 209 tested samples. The kappa coefficient was 0.57, which might be considered good agreement. The specificity was 84.1%, the sensitivity was 84.6%, the positive predictive value (PPV) was 55% and the negative

predictive value was 95.5% [25]. The PCR had a 100% sensitivity reported by two studies with specificities of 93% and 100% [19-26]. Additionally, a retrospective study conducted in the Netherlands reported a 92% sensitivity and a 98% specificity for a PCR targeting the mip gene [27].

In the current study, the UAT for the legionella was positive in 17 samples out of the 100 tested samples for pneumonic patients (17%). While by using respiratory specimen culture technique only 12 samples (12%) were found to be positive. Similar results were shown by Ricci et al., which recorded 40 positive samples for Legionella by UAT technique out of 278 pneumonic patients (14.3%) and 21 samples of them were also positive by Sputum culture while the total positive cases detected by the culture were 35 out of 354 cases (9.9%) [18]. It is apparent from our study that, the positive rate of the UAT (17%) exceeds that for the culture (12%); this was also found by Chen et al. as the positivity rate in pneumonic patients by culture was (0.4%) while by UAT positivity rate for Legionella was (2.7%) [28].

For evaluation of UAT assay, it has a sensitivity of 100%, a specificity of 94.3%, a positive predictive value of 70.6%, a negative predictive value of 100% and an accuracy of 95%. In addition, it has a significant correlation with the culture (Kappa agreement  $0.79 \pm 0.009$ ,  $p$  value  $< 0.001$ ). (Table 3). Similar results were obtained by another study [29]. On the other hand, 5 out of 100 patients (5%) had negative cultures but positive UAT. This might be explained by the fact that, the urinary secretion of the Legionella antigen occurs within 2-3 days after the infection and lasting for months or even year after the end of infection [30]. Another explanation is the occurrence of non-specific signals, possibly due to the presence in urine of immune complexes that interact with the test and give false positive results [31,32]. For this reason, a single testing to assess efficacy is limited in most clinical practice. Combining other methods for simultaneous testing may maximize its accuracy and further evaluation for predicting the effect of UAT results in clinical practice is needed [33].

In the present study both the sensitivity and the negative predictive values of the UAT were 100%. This is due to the usage of Biotest Legionella urine antigen Enzyme Immunoassay (EIA) kit that can detect the antigens of all L.

pneumophila serogroups [34]. Enhanced sensitivity was also achieved by using novel UAT for legionella kit (LAC-116), that can detect serogroups of legionella other than the subgroup 1 [35]. This was in agreement with our results. 100 % sensitivity was also recorded by other study which evaluated UAT for legionella [36]. On the contrast of the previous results several studies recorded sensitivity and negative predictive values less than 80% [21, 37]. **Ahmed et al.** stated that there was a limitation of UAT as a diagnostic tool (sensitivity of 70-80%) and it could miss the non-serogroup-1 cases. Additionally, it concluded that PCR seems to have a higher accuracy among non-invasive testing, but no method alone is accurate enough to safely rule-in or out LD diagnosis [38]. This difference in the sensitivity may be attributed to the Legionella urinary antigen kits used in these studies which were specific for L. pneumophila serogroup-1 antigen only and so there was failure to detect the disease caused by the other serogroup. The L.pneumophila serogroup-1 causes 50-80% of Legionnaires' disease; therefore, 20-50% of the case remain undiagnosed [38, 39].

Timely rapid identification of the causative pathogens is important for adequate antimicrobial therapy especially for pneumonic patients. The studied PCR and UAT techniques showed shorter turnaround time than that for respiratory specimen culture which considered a strong additive for management of these cases.

## Conclusion

In conclusion urinary antigen test for L. pneumophila and q RT PCR testing are promising method that can be used for rapid identification of Legionella infections in pneumonic patients as both techniques correlated well with the gold standard technique, offering the possibility of identifying more positive cases that may be missed by lower respiratory specimen culture method and to rule out infection. Improvement in the diagnosis of legionella will help to better identification of the prevalence of Legionnaire`s disease. This improvement depends on the use of urinary antigen tests capable of detection of different serotypes of legionella; in addition, the development of standardized PCR assays will be major advances in Legionella diagnostics. But these techniques must be interpreted cautiously and hand-in-hand with culture .

**Conflict of interest:**None.

**Funding:** None.

## Ethical considerations:

This study was approved by the Institutional Research Board, faculty of medicine, Zagazig University (approval No. 10374). This was done after consent the patients for their sharing in the study.

## HIGHLIGHTS

- Urinary antigen test for L. pneumophila and q RT PCR testing are promising method that can be used for rapid identification of Legionella infections.
- Improvement in the diagnosis of legionella will help to better identification of the prevalence of Legionnaire`s disease.
- The development of standardized PCR assays will be major advances in Legionella diagnostics. But these techniques must be interpreted cautiously and hand-in-hand with culture .

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