




# Potential false-positive urine *Legionella* enzyme immunoassay test results

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## Abstract

The objective of this study was to identify potential false-positive urine *Legionella pneumophila* (*Legionella*) enzyme immunoassay test results. A total of 107 consecutive patients with positive EIA tests were retrospectively analyzed over a 34-month period. Concurrent blood, urine, and sputum cultures, as well as chest radiographic findings, were reviewed in these patients. Twenty patients (19%) had no radiographic evidence of pulmonary disease despite a positive EIA test. In those 20 patients, 14 also had growth of non-*Legionella* bacteria. Of patients with an infiltrate or opacity on chest imaging, only 27 had *Legionella* sputum cultures obtained, with *Legionella* culture growth occurring in 7 (26%). Nine other patients had negative *Legionella* sputum cultures but the growth of another pathogenic organism in blood, sputum, and/or urine cultures. *Pseudomonas aeruginosa* was the most common organism isolated, found in 20% of patients in the entire cohort. Twenty-five patients (23%) were characterized as having probable false-positive *Legionella* urinary antigen EIA testing, and an additional 17 patients (16%) were characterized as having possible false-positive *Legionella* EIA tests. Our findings suggest that urine *Legionella* EIA tests may lead to a substantial number of cases being misdiagnosed as Legionnaires' disease in patients with non-*Legionella* bacterial colonization or infection.

**Keywords** *Legionella* · Enzyme immunoassay · EIA · Urine antigen testing · Urine alone

## Introduction

*Legionella pneumophila* (*Legionella*) is a facultative intracellular, gram-negative bacillus. This bacterium is the most common etiology of Legionnaires' disease, an acute bacterial pneumonia that was originally identified following an outbreak at a Philadelphia hotel during the 1976 Pennsylvania State American Legion convention [1]. Since its original identification as a cause of community-acquired pneumonia,

subsequent estimates place the annual US incidence of *Legionella* infection at 70 cases per million inhabitants [2]. Although most infections described today occur sporadically and not by the outbreak, mortality rates may range between 10 and 15% of cases [3].

The increasing recognition of *Legionella* as a cause of severe community-acquired pneumonia fueled the development of more rapid diagnostic tests, as compared with traditional culture plate methods. Within several years of the 1976 outbreak, urine antigen testing emerged as an alternative diagnostic modality. Starting as a polyclonal enzyme-linked immunosorbent assay (ELISA) [4], subsequent versions of urine antigen testing included a radioimmunoassay (RIA) [5] and most recently enzyme immunoassay (EIA). These tests are reported to be both highly sensitive and specific for *Legionella* serogroup 1. The Alere manufacturer reports a sensitivity of 97.7% and specificity of 100% [6]. A large meta-analysis of 30 studies reports a pooled sensitivity and specificity of 74% and 99.1%, respectively. However, the suboptimal quality of the included studies and the potential presence of publication bias led the authors to suggest an overestimation of test performance [7].

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Despite these impressive reported performance characteristics of EIA urine *Legionella* antigen testing, concerns have been more recently raised in our institution regarding potential false-positive results. We have suspected false-positive urine EIA tests, particularly in patients with concomitant *Pseudomonas aeruginosa* infections. Thus, this study was designed to analyze and describe the microbiology, including all organisms isolated in blood, sputum, and urine cultures, and confirmation of pneumonia radiographically using chest imaging in all patients with positive EIA urine *Legionella* antigen testing.

## Materials and methods

Patient data was obtained by retrospective chart review of two major hospitals within the Allegheny Health Network (AHN): (1) Allegheny General Hospital, a 631-bed quaternary hospital with 22,000 average annual inpatient admissions, and (2) West Penn Hospital, a 317-bed community-based teaching hospital with 6800 average annual inpatient admissions. All inpatient admissions from January 1, 2015, to October 15, 2017, with positive urine EIA *Legionella* antigen results were included in the analysis. This data was obtained through the EPIC™ electronic health record and further verified with our clinical microbiology laboratory.

Blood, sputum, and urine culture data, as well as chest radiographic imaging, were reviewed when obtained 7 days before and 7 days after the date of a positive urine EIA *Legionella* antigen test.

Urine *Legionella* antigen tests were performed using the Abbott (formerly Alere) Binax™ *Legionella* Urinary Antigen EIA kit, which is an EIA intended to qualitatively detect the presence of *Legionella pneumophila* serogroup 1 antigen in urine as an adjunct to culture for the presumptive diagnosis of past or current Legionnaires' disease. All assays were performed with test samples and controls run in duplicate, where the agreement between samples was within  $0.02 \pm$  standard deviation 0.005. A quality control was performed for each of the implicated runs, and all of these did meet the performance criteria for the test. Outside of normal, expected minor variances, there were no deviations noted in the temperature in the microbiology laboratory during the study period with daily temperatures ranging from 26 to 28 °C, an acceptable range for room temperature incubation in EIA testing.

*Legionella* sputum cultures were plated on Thermo Scientific™ Remel™ non-supplemented buffered charcoal yeast extract (BCYE) agar and Thermo Scientific™ Remel™ BCYE differentiation agar with polymyxin B, anisomycin, and vancomycin for isolation and differentiation of *Legionella* species from clinical specimens. For respiratory secretions, swabs were inserted into the most purulent

portions of the specimen containing blood or mucus, if present. Then the swab was used to inoculate the plate. For bronchial lavage samples, 30–50 mL of fluid was centrifuged at  $1500 \times g$  for 20 min, all but 0.3–0.5 mL of the supernatant decanted and the remaining supernatant vortexed to re-suspend the resulting pellet. Using a sterile Pasteur pipette, two drops of the specimen was then used to inoculate each plate. For each specimen cultures, both the BCYE agar and the BCYE differentiation agar plates were inoculated and incubated for up to 7 days, examining the plates with a dissecting microscope at a magnification of  $\times 20$  and  $\times 50$  beginning at 3 days. A focused light source was used to ensure adequate surface illumination, and all work was conducted in a biological safety cabinet to avoid exposure or cross contamination of specimens. In the event that heavy growth of non-*Legionella* bacteria was present on the BCYE agar and BCYE differentiation agar plates at 24 h, the original sample was reprocessed using an acid-wash treatment. This involved placing 0.1 ml of the specimen into 0.9 ml of potassium chloride acid-wash solution, pH 2.2 (Remel, Inc), and incubating for 5 min prior to inoculating 0.1 ml aliquots each onto BCYE agar, BCYE differentiation agar, and blood agar plates. Finally, a gram stain was also performed with *Legionella* appearing as small gram-negative rods that stain faintly. Again, suspicious colonies were sub-cultured to BCYE agar, BCYE differentiation agar, and blood agar plates for further evaluation. All cultures that were presumptive positive on plated medium were confirmed using a latex agglutination test. The Oxoid *Legionella* latex test uses antibody sensitized blue latex particles which will agglutinate in the presence of specific *Legionella* cell wall antigens to form visible clumps.

Isolation of coagulase-negative *Staphylococcus* species (spp.), *Corynebacterium* spp., *Micrococcus* spp., and *Propionibacterium acnes* from blood and sputum were considered to be contaminants and not included in the data analysis. Sputum *Enterococcus* spp., sputum *Candida* spp., and urine *Candida* spp. isolates were also excluded as they were deemed to represent colonization. Growth of normal respiratory flora in sputum cultures was similarly discounted in this analysis.

For the purposes of this evaluation, patients were categorized as having a probable false-positive *Legionella* urinary antigen EIA result in the following scenarios:

- i) Lack of infiltrate or opacity on chest imaging when both chest plain film and computed tomography (CT) imaging were performed
- ii) Lack of infiltrate or opacity on chest imaging when only chest plain film was performed AND there were no documented pulmonary symptoms AND the patient recovered without receipt of anti-*Legionella* antimicrobial therapy
- iii) *Legionella* sputum culture obtained and negative but with growth of other pathogenic bacteria

Patients were categorized as having a possible false-positive EIA result in the following scenarios:

- i) Lack of infiltrate or opacity on chest imaging when only chest plain film was performed but the patient EITHER had pulmonary symptoms AND/OR received anti-*Legionella* antimicrobial therapy
- ii) *Legionella* sputum culture was not obtained and there was growth of other pathogenic bacteria in blood, sputum, or urine cultures

This study was granted exempt status from the AHN Institutional Review Board as it was deemed a Quality Assessment/Quality Improvement investigation.

### Results

A total of 107 patients had positive urine *Legionella* EIA tests between January 1, 2015, and October 15, 2017, and were included in the analysis. Twenty-five patients (23%) were categorized as having probable false-positive *Legionella* EIA tests. An additional 17 patients (16%) were categorized as

having possible false-positive EIA testing. Of these patients, 13 were categorized as possible false-positive EIA due to the growth of other pathogenic bacteria in blood, sputum, or urine cultures in patients who did not have *Legionella* sputum cultures obtained. Four patients were categorized as having possible false-positive EIA testing due to lack of infiltrate or opacity with only chest plain film performed but had either the presence of pulmonary symptoms and/or received anti-*Legionella* antimicrobial therapy (Fig. 1).

Twenty patients (19%) had no radiographic infiltrates present on chest imaging. Ten of these 20 patients had CT performed without infiltrate or opacity, in addition to unrevealing chest plain film, and were characterized as probable false-positive *Legionella* urinary antigen EIA testing. Of the remaining 10 patients without an infiltrate or opacity on chest plain film, but who did not have chest CT performed, 6 patients did not have pulmonary symptoms and recovered without the receipt of anti-*Legionella* antimicrobial therapy and were also categorized as probable false-positive EIA testing. Additionally, 14 of these 20 patients who did not have an infiltrate or opacity present on chest imaging had another pathogen recovered from blood, sputum, or urine. Thirty-three (31%) of the 107 patient cohort had *Legionella* sputum

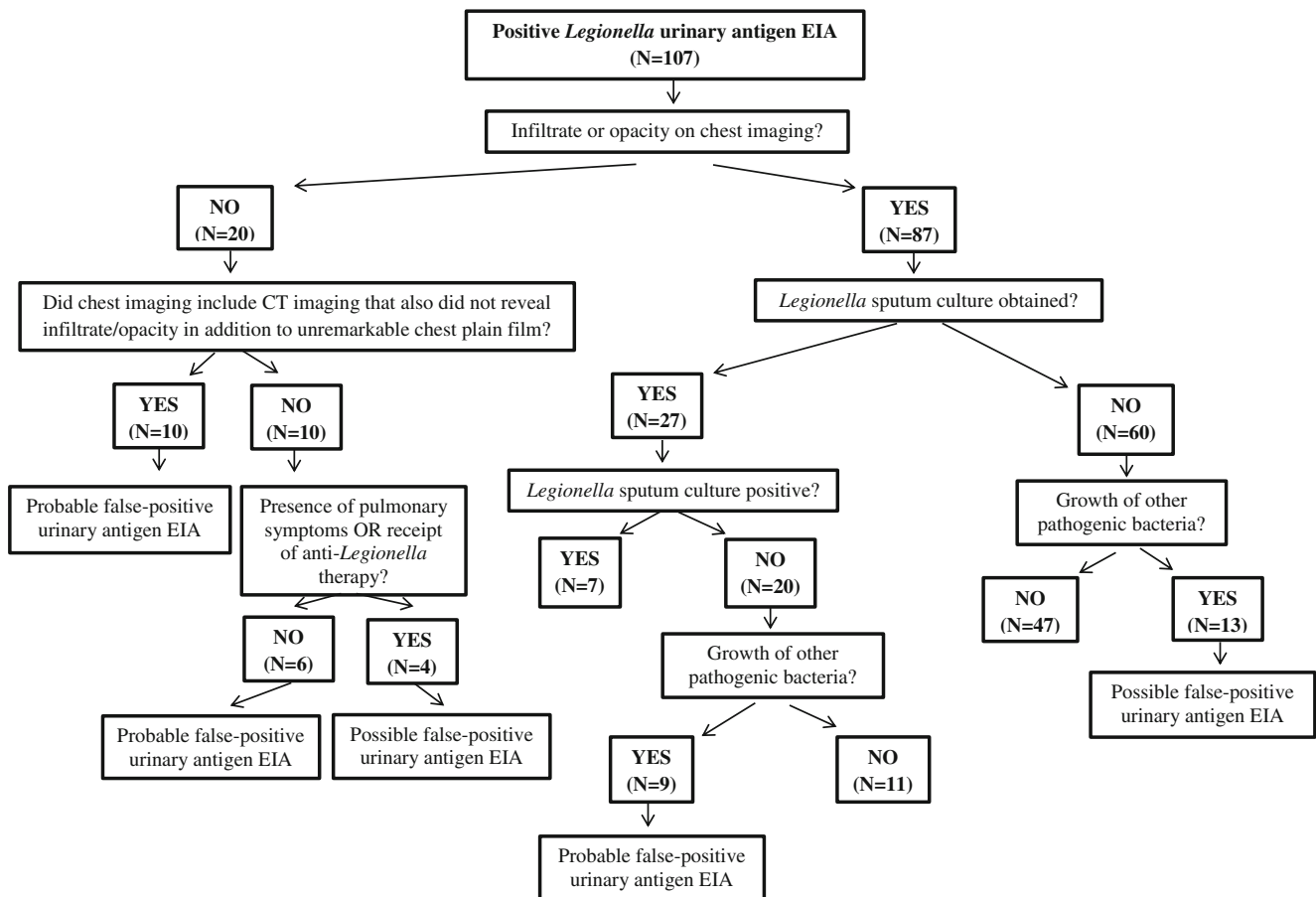


Fig. 1 Definitions for probable and possible false-positive *Legionella* urinary antigen enzyme immunoassay (EIA) testing

cultures obtained, with 27 of these 33 patients having an infiltrate or opacity on chest imaging. Only 7 (21%) of those patients had *Legionella* culture growth. All 7 patients with *Legionella* sputum growth had radiographic infiltrates on pulmonary imaging. Of these 7 patients, 1 patient had concomitant sputum growth of both *Haemophilus influenzae* and *Klebsiella pneumoniae*, and a second patient had sputum growth of group C *Streptococcus*. In the remaining 5 patients in this group, there was no growth of other bacteria in blood, sputum, or urine cultures.

Of the 20 patients with infiltrates or opacities on chest imaging who also had negative *Legionella* sputum cultures, 9 (45%) had growth of pathogenic bacteria from either blood, sputum, urine, or a combination of multiple sites and were deemed to have probable false-positive urinary antigen EIA testing. Additionally, isolation of potential pathogenic organisms was noted in 3 patients who had a negative *Legionella* sputum culture and were without an infiltrate or opacity on chest imaging. *Pseudomonas aeruginosa* was the most commonly identified pathogen, present in 7 of these patients. *Enterobacter cloacae*, *Proteus mirabilis*, *Staphylococcus aureus*, *Providencia stuartii*, *Moraxella catarrhalis*, and *Enterococcus* species comprised the remaining isolates (Table 1).

Of the remaining 60 patients who had an infiltrate or opacity on chest imaging and who did not have a *Legionella* sputum culture collected and performed, 13 had growth of another pathogenic organism isolated from blood, sputum, and/or urine cultures. These were categorized as having possible false-positive urinary antigen EIA testing.

In the entire cohort, *P. aeruginosa* was the most common pathogen isolated. A total of 21 patients (20%) had growth of *P. aeruginosa* from blood, sputum, and/or urine cultures. Seven of these patients had *P. aeruginosa* isolated from blood, nine had *P. aeruginosa* isolated from sputum, and 10 had growth in urine cultures. Overall, of the 49 positive specimens in this study, 26 (53%) grew *P. aeruginosa*.

During the study time period, a new electronic health record was implemented across our health network. Thus, we do not have reliable and accurate data for the total number of *Legionella* urine EIA tests performed in 2015. From January 1, 2016, through October 15, 2017, a total of 4162 *Legionella* urine EIA tests were performed with 88 resulting positive and 4074 resulting negative for a positive rate of 2.1%.

## Discussion

The *Legionella* urine EIA is a rapid, convenient test that is not resource-intensive and may be performed in most clinical settings. While the manufacturer claims an impressive near 100% specificity, our descriptive study adds weight to the argument that this testing modality may have a higher than anticipated false discovery rate in populations with a low incidence of Legionnaires' disease. In our study, 39% of patients were felt to have either a possible or probable false-positive test. A previous report of *Legionella* EIA false-positivity was described in a renal transplant recipient who developed serum sickness in response to antithymocyte globulin derived from rabbit serum [8]. False-positivity has also been reported with the newer immunochromatographic (ICT) assay of the urine

**Table 1** Summary of patients with negative *Legionella* sputum cultures, but growth of non-*Legionella* pathogens

Patient	Blood culture	Specimen Sputum culture	Urine culture	Chest Imaging Infiltrate/opacity present
1	<i>Enterobacter cloacae</i>	No growth	<i>Enterobacter cloacae</i>	No
2	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i> <i>Proteus mirabilis</i>	No growth	Yes
3	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i>	No growth	Yes
4	<i>Pseudomonas aeruginosa</i>	No growth	<i>Pseudomonas aeruginosa</i> <i>Enterococcus</i> species	No
5	No growth	No growth	<i>Pseudomonas aeruginosa</i>	No
6	No growth	No growth	Mixed gram-negative rods	Yes
7	No growth	<i>Staphylococcus aureus</i>	No growth	Yes
8	No growth	<i>Staphylococcus aureus</i>	<i>Enterobacter cloacae</i>	Yes
9	No growth	<i>Pseudomonas aeruginosa</i> <i>Providencia stuartii</i>	No growth	Yes
10	No growth	<i>Pseudomonas aeruginosa</i>	No growth	Yes
11	No growth	<i>Moraxella catarrhalis</i>	No growth	Yes
12	No growth	No growth	<i>Pseudomonas aeruginosa</i> <i>Proteus mirabilis</i>	Yes



antigen test, BinaxNOW®, in a patient with disseminated *Nocardia* infection [9]. *Legionella* sputum cultures were negative in both of these cases. Hypothesized mechanisms for these occurrences include interfering peptides in the former case, and cross-reacting lipopolysaccharide antigens derived from other pathogenic bacteria in the latter. While boiling, centrifugation, and resultant urine supernatant testing have been reported to eliminate false-positive EIA reactions due to interfering antibodies, this strategy was not reported effective in the *Nocardia* patient case [9–11].

Our study suggests that other pathogenic bacteria, particularly *P. aeruginosa*, but potentially also members of the Enterobacteriaceae family of gram-negative bacilli, may generate false-positive urine *Legionella* EIA test results. Given the widespread use of urine EIA testing, false-positive results and a high false discovery rate could inflate regional reporting of *Legionella* infection rates. Furthermore, inaccurate *Legionella* diagnoses could result in increased patient exposure to unnecessary antibiotic agents, including macrolides and fluoroquinolones, and their resultant potential adverse effects. Additionally, false positive results may trigger costly and protracted resource-intensive investigations by health departments when cases may be considered hospital-acquired. Larger studies that can both further define the relationship between non-*Legionella* bacterial infection and false-positive urine EIA test results, as well as corrective interventions, are sorely needed.

Our study has several important limitations. First, a paucity of patients in our total cohort had sputum specimens sent for *Legionella* culture, which limits our ability to more fully describe the total number of patients who had discordant results between urinary antigen testing and *Legionella* sputum culture. Additionally, during this time period, we only utilized Binax™ EIA urinary antigen testing. It is possible that the use of BinaxNOW® ICT testing would have produced fewer positive tests. However, given the nature of our study, we are unable to analyze this. Also, we were unable to determine if any of our samples, which resulted in suspected false-positive *Legionella* EIA urinary antigen testing, would have yielded a negative result after boiling, which has been postulated to reduce interfering antibodies that may lead to false-positive EIA reactions [11]. In our analysis, if there were growth of other pathogenic bacteria, we categorized these as possible false-positive urinary antigen EIA testing, and if there were growth of other pathogenic bacteria with a negative *Legionella* sputum culture, we categorized these as probable false-positive urinary antigen EIA testing. We did not exclude the possibility of co-infection in these scenarios, but were unable to confirm these definitely as false-positives given our inability to perform further testing upon these specimens in our retrospective study. Also, we characterized patients without an infiltrate or opacity on chest imaging when both chest plain film and CT imaging were performed as probable

false-positive EIA tests. However, a positive urinary antigen test may have indicated less severe, non-pneumonic Pontiac fever rather than the typical Legionnaires' disease. Lastly, we were not able to provide definitive evidence of false-positive EIA tests. Given the retrospective nature of the analysis, we were unable to prove false-positive tests using an alternative test methodology.

Given our findings and limitations, coupled with data suggesting that false-positive tests due to non-*Legionella* bacteria may convert to negative after boiling, a future prospective study includes the collection and analysis of urinary and sputum specimens from patients that yield positive *Legionella* urine EIA tests. This will allow for a more thorough comparison of testing with the current EIA with and without boiling, as well as comparing the Binax™ EIA to the BinaxNOW® ICT test, *Legionella* sputum culture with BCYE agar and BCYE differentiation agar, and *Legionella* polymerase chain reaction from sputum. Additionally, for microbiology laboratories utilizing the urine EIA test, strong consideration should be given to repeating all positive urine EIA tests after boiling or confirming with another testing methodology.

In conclusion, urinary antigen testing has greatly enhanced the ability to diagnose pneumonia resulting from *Legionella*. Despite the published report of greater than 99% specificity with these modalities, recent reports have indicated false-positive EIA results. One hypothesis for this false-positive result is the excess protein-complex in the urine, a future study planned in our group. In the present study, we describe numerous examples of suspected false-positive *Legionella* urinary antigen results using BINAX™ EIA testing, with 23% and 16% of the total cohort being characterized as having probable and possible false-positive EIA test results, respectively. These findings highlight the overlooked possibility of a substantial number of cases that are misdiagnosed as Legionnaires' disease when utilizing a test with high specificity when deployed in a population with a low incidence of disease. Thus, these findings indicate the need for more rigorous, prospective studies to directly compare and evaluate potential false-positive rates of *Legionella* urinary antigen testing both EIA and ICT methodology.

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