

A Simple, Specific, and Rapid Lateral-Flow Immunochromatographic Test Method for Detection of *Legionella pneumophila* in Water Samples

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Abstract. To control and determine the cause of clustered cases of respiratory disease, a means of rapid assessment of contamination of environmental water samples with *Legionella pneumophila* (*Lp*) was developed. An *Lp* specific antibody was identified that functions in a lateral flow immunoassay. Colloidal gold particles coated with the antibody recognizes *Lp* and coupled with biotin-labeled antibody capture the bacterial cells and migrate to a test line, which indicates the presence of the pathogen that is visible within 15 minutes. The sensitivity of the assay is 10⁵ CFU/ml demonstrated by detection of *Lp* in water samples. Cross reaction wasn't observed with three other bacterial species: *B. anthracis*, *E. coli* and *S. aureus*, even in the background of a turbid field-collected water sample. These lateral flow immunochromatographic assays for *Lp* are selective, relatively sensitive, extremely rapid, and easily performed outside of the laboratory.

Keywords: *Legionella pneumophila*, Lateral-Flow, field test.

1. Introduction

Legionella pneumophila (*Lp*) is the bacterial pathogenic agent of Legionnaires' disease, which was first identified in 1976. Since then, more than 50 species of *Legionella* and over 70 serogroups have been identified. Although many species have been reported that can cause infectious disease in humans^[1], serogroup 1 of *Lp* accounts for more than 90% of legionellosis cases. *Lp* is widespread in freshwater environments, man-made water systems, soil, sediment, and even in salt water^[2-5]. Aerosols contaminated by *Lp* enter the lungs and can cause an acute form of pneumonia known as Legionnaires' disease, or a milder form of self-limited infection called Pontiac fever.

The "gold standard" for the detection of *Legionella* in water is based on a culture method defined as ISO 11731. The assessment of risk associated with *Legionella* is performed using culture-based methods, which is essential for identifying and typing *Legionella* strains during epidemics. However, culture-based methods have some limitations: long incubation times up to 10 days are required in BSL-2 laboratory conditions. Microbial contamination in the water may inhibit *Legionella* growth, and the bacteria cannot be detected when it is present in the viable but nonculturable (VBNC) state in the environment. Therefore, the culture-based method is not suitable for real-time detection and risk assessment when outbreaks of respiratory disease occur. Since the *Legionella* bacteria pose a great health threat to humans, the rapid detection of *Legionella* in the aqueous environment has become a priority in preventing and controlling the disease.

Lateral flow immunochromatographic assays (LFIA) are inexpensive and easy to use diagnostic methods which make them ideal for use in non-lab-dependent field tests. The first tests of LFIA were made

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for the detection of human chorionic gonadotropin (hCG). Today LFIA techniques are widely used for monitoring pregnancy, detecting infectious disease organisms, analyzing drugs of abuse, and measuring other analytes important to human physiology. There are many LFIA test strips designed to test bacterial contamination: *Shigella dysenteriae*, *Francisella tularensis*, anthrax, syphilis, herpes simplex virus, and other infectious disease pathogens or antigens^[6-11]. In the present work we describe the development and evaluation of a LFIA test method for the rapid detection of *Legionella* in water samples.

2. Materials and Methods

2.1. Test Procedure of the *Lp* LFIA

Samples were loaded onto the Generic Rapid Assay Device, gRAD, from Rapid Assays, Denmark, which is a nitrocellulose based assay device with immobilized streptavidin (test line) and a species specific anti-IgG antibody at the control line (Figure 1A). The 40nm gold particles used were from Rapid Assays. The antibody to *Lp* for the sandwich immunoassay is a rabbit polyclonal # 20-LR45 from Fitzgerald Industries, MA, USA. The antibody was labeled with biotin using Thermo Scientific, IL, USA EZ-Link NHS-PEG4-Biotinylation kit, # 21455. The test analyte of inactivated *Lp* and Inactivated *Bacillus anthracis* vaccine strain were from the Critical Reagents Program Antigen Repository, UT, USA.

As shown in Figure 1B, the detection reagent (complexes of biotin labeled target specific IgG antibody plus colloidal gold coated with the specific antibody) and the water sample (or analyte) were mixed, then loaded onto the sample pad of gRAD lateral-flow test strip. After running through the strip, the biotin labeled antibody-target bacteria-colloidal gold coated antibody complexes were trapped by streptavidin on the test line. A visible red to purple line was formed there upon binding of the gold particles/analyte/biotinylated antibody complexes. The excess colloidal gold coated antibody was trapped by the anti-IgG antibody at the control line. In 10-15 minutes, if analyte (specific bacteria) was present, both lines were visible, indicating that the test was valid (Figure 1C). In the present test, 50 μ L raw samples and a dilution series of different concentrations of *Lp* were mixed with an addition of an equal volume of detection reagent, and then loaded on the gRAD to run the LFIA.

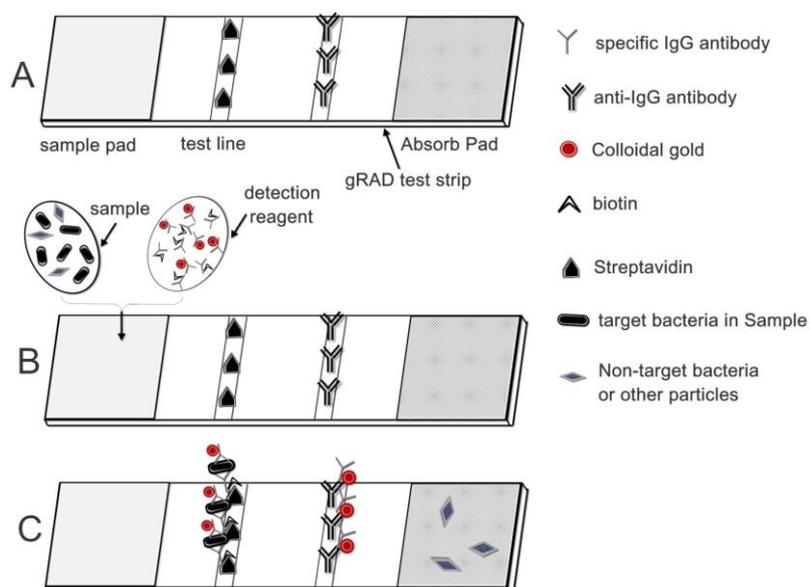


Fig. 1: Schematic illustration of the gRAD lateral-flow assay

2.2. Conjugation of *Lp* Antibody with Biotin

Biotinylation of rabbit anti-*Lp* polyclonal antibody was performed according to the instructions for the EZ-link biotinylation kit. After biotinylation of antibody, excess biotin was removed using desalting columns and the level of biotin incorporation measured using the HABA binding assay with an absorbance shift at 500 nm.

2.3. *Lp* Antibody and Colloidal Gold Particle Coating Procedure

Preliminary experiments using 20nm and 40nm colloidal gold showed that the 40nm colloidal gold and antibody conjugates are more stable and less prone to precipitation, so the 40nm colloidal gold was chosen for the present LFIA test strip design. 0.5 ml of colloidal gold was dispensed into 10 eppendorf tubes; the pH adjusted to 5.4, 6.6, 7.3, 7.8, 8.2, 8.4, 8.8, 9.2, 9.6, and 10.1, respectively, using reagents supplied in the gold colloid conjugation kit (RapidAssays, Denmark). Rabbit anti-*Lp* antibody was added to a final concentration of 30 µg/mL, vortexed for several seconds on a low speed vortexer and incubated at room temperature for 30 minutes. A deepening purple color or black precipitate in some tubes indicated that the antibody is below its iso-electric point (pI) and that the particles were incompletely coated. To test for complete coating, 10 µL of coated colloidal gold was mixed with 10 µL 1M NaCl. Colloidal gold with incomplete coating fall out of solution and turn black while completely coated particles will remain stable and remain red in color.

2.4. Optimization of Colloidal Gold Amount

Analysis of the final results of LFIA is judged by visualizing the red or purple color of the detection and control lines with the naked eye. The amount of the colloidal gold utilized is critical and directly related to the color shades of the final result. Since 40nm colloidal gold has a maximum absorption peak (OD) under 540 nm light, the amount of gold particles with OD₅₄₀ of 0.1, 0.5, 1.0, and 2.0 OD was determined using the DU-40 spectrophotometer (Beckman, U.S.A).

2.5. Sensitivity Test

Inactivated *Lp* was serially diluted ten-fold in phosphate buffered saline (PBS: 100 mM sodium phosphate, 150 mM NaCl pH7.2) from 1×10^8 to 1×10^3 CFU/ml, then 50 µL of this analyte was mixed with the detection reagent to test the sensitivity of LFIA test strip.

2.6. Specificity Test

Inactivated *Bacillus anthracis* vaccine strain (BA), *Escherichia coli* (EC) and *Staphylococcus aureus* (SA) were used to challenge the specificity of *Lp* LFIA test strip. The three bacterial suspensions were in PBS buffer at about 10^8 CFU/ml. 50 µL was mixed with the detection reagent and added to the LFIA test strip.

2.7. Surface Water Spiked Sample Test

Surface water with a turbidity of about 5 NTU collected near Seattle, USA was utilized as the raw sample, and different concentrations of inactivated *Lp* and other bacteria mixtures were inoculated into the surface water as the spiked samples to test the discrimination power of *Lp* LFIA test strip.

3. Results

3.1. Optimization of Detection Reagent

For the detection reagent, which is a mixture of biotin labeled rabbit anti- *Lp* polyclonal antibody and colloidal gold particles coated with the same unlabeled polyclonal antibody; the biotinylated antibody is at a concentration of 1.25 mg/mL with the biotin: protein molar ratio at 1.26:1. The optimal colloidal gold antibody conjugating pH is 6.6 which are influenced by the pI of the antibody. The colloidal gold amount used in the final detection reagent mixture that has the best performance has an OD₅₄₀ of 0.5 with a strong control line and an absent test line.

3.2. Sensitivity

In Figure 2, the sensitivity of the LFIA is demonstrated. Increasing concentrations of analyte from 1×10^3 CFU/ml to 1×10^8 CFU/ml was mixed with the optimized concentration of detection reagents as outlined in the section above. The aggregates were applied to the lateral flow devices and test lines evaluated. When the concentration of *Lp* is less than 1×10^5 CFU/ml, it was difficult to visualize a positive detection line with the naked eye. Therefore the cutoff for the test line to be detected by this method is with the analyte concentration at 1×10^5 CFU/ml (Figure 2, panel C). Since 50 µL of a 1×10^5 CFU/ml analyte suspension is used, the lowest detection limit is 5 000 CFU.

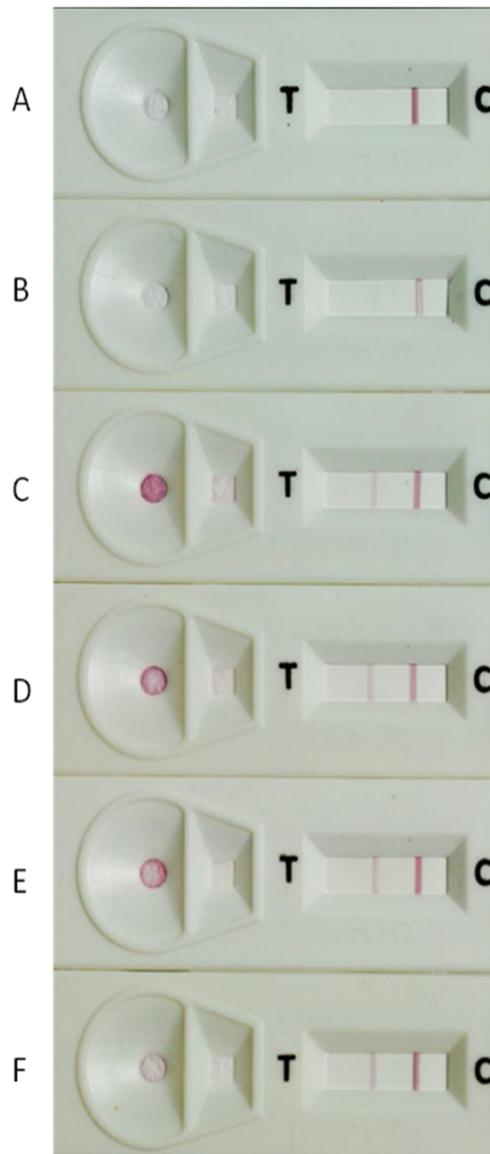


Fig. 2: Sensitivity of the gRAD lateral-flow assay to analyte concentrations.
 A: 1×10^3 CFU/ml, B: 1×10^4 CFU/ml, C: 1×10^5 CFU/ml,
 D: 1×10^6 CFU/ml, E: 1×10^7 CFU/ml, F: 1×10^8 CFU/ml

3.3. Specificity

No test lines are observed at the lateral-flow test strips with *BA*, *EC* and *SA*. These bacteria are examples of spore-forming, gram-negative and gram-positive bacteria, respectively. When the LFIA tests are conducted with the reagent concentrations as determined in the previous two sections, good specificity is achieved due to the anti-*Lp* polyclonal antibody.

3.4. Surface Water Spiked Sample Test Results

In Figure 3, we demonstrate that addition of an environmental water sample does not skew the results, and *Lp* is still detectable at 1×10^5 CFU/ml. There is no test line observed with the raw surface water (panel A), it means that there is no *Lp* in the water or that it is below the detection limit. Panel B shows detection of 1×10^5 CFU/ml *Lp* in surface water. Addition of *BA* does not alter detection of *Lp* (panel C), and addition of *BA*, *EC* and *SA*, all at 10^8 CFU/ml, does not affect detection in surface water (panel D). LFIA test strips show good discriminatory power within the context of surface water with a high background of other bacterial species.

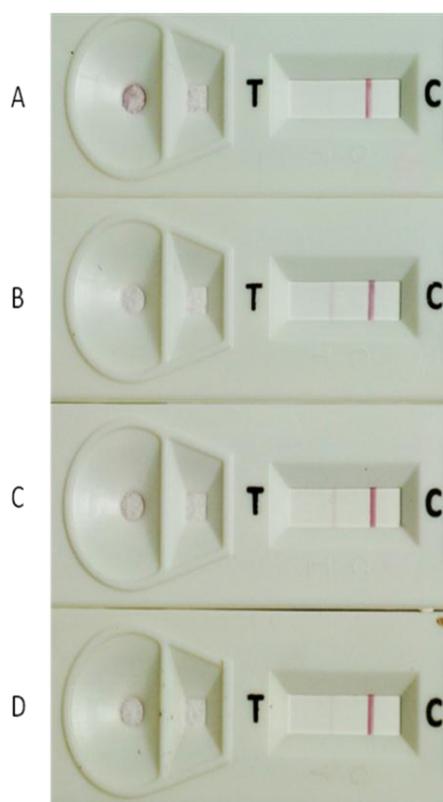


Fig. 3: Surface water spiked sample test results.

A: surface water (SW), B: *Lp* spiked in SW at 1×10^5 CFU/ml, C: 1×10^5 CFU/ml *Lp* and 1×10^8 CFU/ml *EC* in SW, D: 1×10^5 CFU/mL *Lp* plus 1×10^8 CFU/mL each of *BA*, *EC*, *SA* combined in SW.

4. Discussion

LFIA is an immunological-based method; in this application the targets of detection are antigens exposed on the surface of the bacterial cells. LFIA detects both living and dead *Legionella*. Like LFIA, PCR based detection methods also cannot discriminate dead cells from live cells. Conversely, culture-based methods only detect the cultivable bacterial cells, and generate CFU counts. Methods used prior to culturing *Legionella* (such as pretreatment with acid or heat) reduce its cultivability on selective medium and thus the actual numbers may be underestimated. Additionally, *Legionella* can enter into a dormant non-dividing VBNC state under environmental pressures and still possess pathogenicity^[12]. Therefore, the culture method underestimates the risk of health posed by *Legionella* in environmental samples.

In the present test of *Legionella* detection; the sensitivity of LFIA is 5 000 CFU and 10^5 CFU/ml. Though the detection limit is less sensitive than culture-based and PCR-based methods, where detection limits are 10 CFU/ml and 10^2 - 10^4 CFU/ml respectively^[13-14], this type of assay can be formulated to be ready to use, has simple non-complicated operating procedures, requires less time (only 15 minutes), and is not dependent on well trained personnel and laboratory instrumentation. A once commercially available lateral flow assay to detect species of *Legionella* such as *Lp* and *Legionella anisa* (Duopath, Merck) may be qualitative rather than quantitative^[15], performs only down to 10^7 CFU/ml for the former species^[16], and lacks the sensitivity to be usable in the field. We demonstrate detection down to 10^5 CFU/ml in simulated environmental conditions. That level of sensitivity in a lateral flow immunoassay for *Legionella* will potentially supply a rapid survey method for on-site screening and emergency diagnosis when a suspected waterborne disease outbreak occurs.

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6. References

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