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Increased sensitivity of a direct fluorescent antibody test for *Legionella pneumophila* in bronchoalveolar lavage samples by immunomagnetic separation based on BioMags

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Abstract

In the present study, immunomagnetic separation of *Legionella pneumophila* from mock bronchoalveolar lavage (BAL) fluid samples, which were artificially spiked with *L. pneumophila*, and culture positive patient BAL fluid samples, was achieved using BioMags (superparamagnetic particles) loaded with purified rabbit immunoglobulin G specific for *L. pneumophila*. Bacteria binding onto BioMag-immunomatrix were directly stained with a *L. pneumophila* species-specific DFA reagent and examined under a fluorescence microscope. BioMag-based immunomagnetic separation (BIMS) followed by DFA staining (BIMS–DFA) could correctly identify all the 20 (100%) BAL samples which were spiked with low numbers (2×10^2 CFU) of *L. pneumophila*. Cultures could be recovered from 15 (75%) of these 20 spiked BAL samples, 5 (25%) of the samples failed to yield positive cultures. Both culture and BIMS–DFA methods showed 100% positive results when spiked BAL samples containing high bacterial load (10^4 CFU) were tested. The findings with true patient culture positive BAL specimens which were examined retrospectively indicated that BIMS–DFA is significantly more sensitive for detecting *L. pneumophila* than conventional cytospin method of DFA staining (cytospin–DFA). Out of the 25 culture positive BAL specimens tested, 7 (28%) proved negative by cytospin–DFA whereas BIMS–DFA correctly detected all the 25 (100%) specimens. It is suggested that the BIMS–DFA procedure increases the sensitivity of DFA testing for *L. pneumophila* in large volume samples such as BAL fluids.

Keywords: BioMags; Immunomagnetic separation; Bronchoalveolar lavage; *Legionella pneumophila*; Direct fluorescent antibody; Sensitive detection

1. Introduction

Legionella pneumophila (*L. pneumophila*), a rod-shaped bacterium, is the causative agent of both Legionnaires disease (a potentially fatal multisystem disease involving pneumonia) and Pontiac fever, a self-limited flu-like illness (Fraser et al., 1977; Glick et al., 1978). Of the several known *Legionella* species, *L. pneumophila* (comprising multiple serogroups) is accepted as the principal cause of human outbreaks of legionellosis with serogroup 1 (SG 1) bacterium responsible for a majority (79–90%) of all culture confirmed cases (Marston et al., 1994; Yu et al., 2002).

The need for rapid detection of *L. pneumophila* in clinical samples is of crucial importance, as prompt initiation of antimicrobial therapy improves the outcome of the disease (Heath et al., 1996). A number of methods have been used to detect/identify this pathogen in different types of clinical specimens. The conventional culture method using selective media is accepted as the gold standard for the detection of *L. pneumophila* (Fields et al., 2002). Culture isolates are mostly identified by serologic tests using commercially available polyclonal antisera (Thacker et al., 1985). The disadvantages of culture are the delay in obtaining results because of long incubation periods of 2–6 days (Grimont, 1986) and low sensitivity of culture from respiratory samples (Fields et al., 2002).

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Among the currently available rapid molecular diagnostic methods for detecting *L. pneumophila* in clinical samples, DNA probes for *in situ* hybridization have been reported to have sensitivities of 30 to 75% (Edelstein, 1987; Fain et al., 1991; Fields et al., 2002; Wilkinson et al., 1986). The other nucleic acid based techniques, such as polymerase chain reaction (PCR) and real-time PCR combined with probe hybridization are rapid and reliable for detecting *L. pneumophila* under clinical settings (Ballard et al., 2000; Hayden et al., 2001; Kessler et al., 1993; Rantakokko-Jalava and Jalava, 2001; Reischl et al., 2002; Wilson et al., 2003). However, for both real-time PCR and conventional PCR assays, complex clinical samples such as bronchoalveolar lavage (BAL) fluids may pose problems with inhibition of Taq polymerase enzyme as well as through template contamination which is likely to yield false negative results (Jaulhac et al., 1998). Commercially available enzyme-linked immunosorbent assays (EIAs), which enable the detection of soluble urinary antigen, primarily detect *L. pneumophila* SG 1 antigen (Kashuba and Ballow, 1996). Obviously, a negative urinary antigen test result does not rule out infection caused by other serogroups (Nguyen et al., 1991; Fields et al., 2002). In a majority of clinical laboratories, the most commonly used method for detecting *L. pneumophila* is either culture or direct fluorescent antibody (DFA) staining. DFA staining is a most rapid and convenient method, which is performed either by fluorescein-isothiocynate (FITC)-conjugated monoclonal or polyclonal antibodies to *L. pneumophila* (Edelstein et al., 1985). The sensitivity of DFA testing has ranged from 25–85% with a specificity greater than 95% (Edelstein, 1987). It has been reported that the culture method is more sensitive than DFA testing, by a factor of 1.2 to 4 (Edelstein, 1984). Because of the apparent advantages of DFA test for detecting *L. pneumophila*, the present study evaluated whether the use of immunomagnetic concentration and separation of the bacteria in bronchoalveolar lavage (BAL) fluids will improve the sensitivity of the DFA assay.

Immunomagnetic separation (IMS) is a powerful technique which has been used extensively for the enrichment and separation of a wide variety of pathogenic microorganisms from complex matrixes (foods, environmental waters and clinical materials) before subsequent detection is performed (Cudjoe et al., 1993; Olsvik et al., 1994; Safarik et al., 1995). The major advantage of IMS is the rapid separation and simultaneous concentration of captured targets from surrounding milieu when placed in a magnetic field. The technique is however limited by the requirement for specific antibodies with high avidity and affinity for surface epitopes on the targeted organisms. Several types of paramagnetic beads/particles with different sizes, shapes and chemistries are commercially available. An earlier study (Yanez et al., 2005), reported the application of polystyrene paramagnetic beads (Dynalbeads M-280; Dynal Biotech, Norway) for IMS of *L. pneumophila* SG 1 bacteria from seeded environmental water samples and their subsequent detection by conventional culture and real-time PCR methods. In the present study, we used small (1 μm), non-uniform (irregular shaped) silanized paramagnetic particles called BioMags (Polysciences, Inc., Eppelheim,Germany) for IMS and concentration of *L. pneumophila* from BAL specimens and the direct and specific visualization of intact bacteria while still bound to the BioMag-immunomatrix by a monoclonal DFA reagent.

2. Materials and methods

2.1. Bacterial strains

L. pneumophila strains representing SG 1 through 8 and SG 10, *L. micdadei* and *L. jordanis* originating from American Type Culture Collection (ATCC, USA) were used in this study (Table 1). Different isolates belonging to *L. pneumophila* SG 1 included Philadelphia-1, Olda, Pontiac, Knoxville and Bellingham. Frozen stocks of these isolates were obtained from the Centers for Disease Control, Atlanta, Georgia, USA. These isolates displayed different reactivity patterns (subtypes), when tested with a panel of monoclonal antibodies against *L. pneumophila* SG 1 (Sethi, 1985). The cultures were maintained as frozen stocks at −20 °C. The strains were grown on buffered charcoal yeast extract agar supplemented with ketoglutarate (BCYE) agar (Heipha Diagnostics, Eppelheim, Germany) for 3 days at 37 °C and harvested in phosphate buffered saline (PBS, pH 7.4).

2.2. Enumeration of bacteria

The number of colony forming units (CFU) was determined by the enumeration of the colonies formed on BCYE agar plates. About 4–5 colonies from 3 day old cultures were suspended in 1 ml of sterile PBS, pH 7.4 and adjusted turbidimetrically to 10^7 bacteria per ml (optical density at 660 nm, 0.2). For calculating the exact number of *L. pneumophila* in the suspension, 10-fold serial dilutions were prepared in PBS and 100 μl aliquots of each

Table 1
Recovery by BioMag-based immunomagnetic separation from culture suspensions of different *L. pneumophila* strains/isolates

Species	Serogroup	Strain (or isolate)	Source recovery [a]	
L. pneumophila	SG 1	Philadelphia-1	(ATCC 33152)	55.8 (4.2)
	SG 1	Olda	(CDC)	60.2 (3.6)
	SG 1	Pontaic	(CDC)	49.8 (4.4)
	SG 1	Bellingham	(CDC)	68.4 (2.9)
	SG 1	Knoxville	(CDC)	65.2 (3.4)
	SG2	Togus 1	(ATCC 33154)	48.8 (4.1)
	SG3	Bloomington 2	(ATCC 33155)	51.7 (3.6)
	SG 4	Los Angeles 1	(ATCC 33156)	53.2 (2.5)
	SG 5	Cambridge 2	(ATCC 33216)	60.2 (3.2)
	SG 6	Chicago 2	(ATCC 33215)	44.8 (3.5)
	SG 7	Chicago 8	(ATCC 33823)	51.4 (2.6)
	SG 8	Concord 3	(ATCC 35096)	33.7 (2.7)
	SG 10	Leiden 1	(ATCC 43283)	30.6 (2.9)
L. micdadei		Tatlock	(ATCC 33217)	0
L. jordanis			(ATCC 33623)	0

Abbrevations: ATCC, American Type Culture Collection; CDC, Centers for Disease Control.

[a] Values represent mean (standard deviation) percent recovery from triplicate experiments. Initial No. of organisms seeded = 2×10^3 CFU/ml.