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Contribution of Amoebic Coculture to Recovery of *Legionella* Isolates from Respiratory Samples: Prospective Analysis over a Period of 32 Months

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We evaluated the contribution of amoebic coculture to the recovery of *Legionella* spp. from 379 respiratory samples. The sensitivity of axenic culture was 42.1%. The combination of axenic culture with amoebic coculture increased the *Legionella* isolation rate to 47.1%. Amoebic coculture was particularly efficient in isolating *Legionella* spp. from respiratory samples contaminated with oropharyngeal flora.

Legionella spp. are facultative intracellular Gram-negative bacteria that are ubiquitous in natural and man-made aqueous environments, in which they survive as free-living bacteria or, more commonly, as intracellular forms in amoebae (1). When humans inhale contaminated aerosols, legionellae can infect and replicate within lung macrophages and cause a severe pneumonia referred to as Legionnaires' disease (LD).

Urinary antigen detection is the first-line diagnostic test, although this test is limited to *Legionella pneumophila* serogroup 1 (Lp1) (7). Molecular techniques improve LD diagnosis by detecting other serogroups and species. Nevertheless, isolation of *Legionella* strains is required to perform further epidemiological investigations. The sensitivity of axenic culture ranges from 15% to 90%, depending on the *Legionella* inoculum, the level of contamination of the samples with oropharyngeal flora, the prior use of antibiotics, and the experience of the laboratory members (2, 4). Several authors have described amoebic coculture as a method to recover *Legionella* spp. from culture-negative specimens (3, 8, 10–12). However, large-scale studies evaluating the benefit of amoebic coculture in routine laboratory practice are lacking.

In this work, we evaluated the contribution of amoebic coculture to the recovery of *Legionella* spp. from 379 respiratory samples collected over a period of 32 months.

This prospective study included 348 patients with suspected LD who were admitted to 98 French hospital facilities from April 2008 to November 2010. An LD case was defined as a patient with clinical and/or radiological findings compatible with pneumonia and at least one positive test for *Legionella* spp.; the tests conducted were detection of urinary antigen by an immunochromatographic test and axenic culture and amoebic coculture of a respiratory sample. A total of 379 pulmonary samples consisting of 168 sputum, 48 tracheobronchial aspiration (TBA), and 163 bronchoalveolar lavage (BAL) samples sent to the French National Reference Center of *Legionella* for diagnosis or epidemiological investigations were collected and processed by both axenic culture and amoebic coculture.

Axenic culture was performed upon sample arrival at the laboratory. The samples were liquefied using dithiothreitol (Sputasol; Oxoid, Dardilly, France) if necessary. One hundred microliters was inoculated onto five plates: buffered charcoal yeast extract (BCYEα medium; Oxoid); BCYE supplemented with cefamandole, polymyxin, and anisomycin (BMPA medium; Oxoid) (two plates); BCYE supplemented with glycine, vancomycin, polymyxin B, and cycloheximide (GVPC medium; bioMérieux, Marcy l'Etoile, France); and tryptone soy sheep blood agar (TSS medium; bioMérieux). The plates were incubated for 10 days at 35°C in an aerobic atmosphere (BCYEα, BMPA, and TSS media) or in a 2.5% CO_2 atmosphere (BMPA and GVPC media). Acid decontamination (HCl, pH 2, 30 min at room temperature; neutralized with NaOH, pH 11) or heat treatment (50°C, 30 min) was performed 24 h later for cultures contaminated with oropharyngeal flora.

Amoebic coculture was performed weekly, and the samples were stored at +4°C until coculture. *Acanthamoeba polyphaga* (Linc AP-1; from April 2008 to April 2009) or *Acanthamoeba castellanii* (ATCC 30234; from May 2009) was grown in peptone-yeast extract-glucose medium at 30°C (8). Amoebae were suspended in Page's amoebic saline (PAS) buffer, and 10^6 amoebae were distributed into each well of a six-well tissue culture plate (BD Falcon; Becton Dickinson, Le Pont-de-Claix, France). One milliliter of the sample was mixed with sterile distilled water (3 ml) to disrupt the cells and centrifuged (1,000 rpm, 5 min). The supernatant was removed and centrifuged (10,000 rpm, 10 min). The pellet was suspended in PAS buffer (200 μl) and inoculated into an amoeba culture. The plate was centrifuged (1,500 rpm, 20 min) and then incubated at 30°C. Three days after inoculation, the plate was gently shaken to suspend the amoebae. One hundred microliters of the infected amoebic suspension was subcultured on BCYE and BMPA media incubated for 10 days at 35°C in a 2.5% CO_2 atmosphere. One hundred microliters was inoculated onto a second amoebic plate. After a 3-day incubation of the sec-

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TABLE 1 Isolation of *Legionella* strains from 240 samples from patients with LD by axenic culture and amoebic coculture

Axenic culture result	No. (%) of amoebic cocultures		
	Positive	Negative	Total
Positive	69[a]	32[b]	101 (42.1)
Negative	12[c]	127	139 (57.9)
Total	81 (33.8)	159 (66.2)	240

[a] 67 Lp1, 1 Lp3, and 1 Lp8.
[b] 32 Lp1.
[c] 11 Lp1 and 1 Lp8.

TABLE 2 Sensitivities of axenic culture, amoebic coculture, and a combination of the two according to the sample type

Sample type (*n*) or parameter	% Sensitivity (*n*)		
	Axenic culture	Amoebic coculture	Combination of axenic culture and amoebic coculture
BAL fluid (53)	64.2 (34)	50.9 (27)	66.0 (35)
TBA (37)	45.9 (17)	27.0 (10)	51.4 (19)
Sputum (150)	33.3 (50)	29.3 (44)	39.3 (59)
Total (240)	42.1 (101)	33.8 (81)	47.1 (113)
P value[a]	0.001	0.011	0.003

[a] Chi-square test.

ond plate, 100 μl of the suspension was subcultured as previously described.

Identification of *Legionella* spp. was performed by latex agglutination (Oxoid, bioMérieux); the Lp1 isolates were typed using Dresden monoclonal antibody (MAb) subgrouping (6) and sequence-based typing (5, 9).

Among 348 patients, 222 cases of LD corresponding to 240 samples were confirmed. The *Legionella* urinary antigen assay was positive in 217 cases (sensitivity, 98%). A total of 113 *Legionella* isolates were recovered by axenic culture and/or amoebic coculture (Table 1). Axenic culture isolated 101 *Legionella* strains (99 Lp1, 1 Lp3, 1 Lp8), yielding a sensitivity of 42.1%. Amoebic coculture demonstrated lower sensitivity than axenic culture (33.8% versus 42.1%, $P = 0.004$; chi-square test). Performing the amoebic coculture weekly and refrigerating and pretreating the samples may have impaired *Legionella* viability and reduced the actual coculture isolation rate. However, amoebic coculture recovered 12 additional strains (11 Lp1, 1 Lp8) and increased the global isolation rate to 47.1%. The 12 corresponding samples were all highly contaminated with oropharyngeal flora, in comparison to 12% (8/69) of the samples recovered by both methods and 19% (6/32) of the samples recovered by axenic culture only. These results suggest that amoebae supporting *Legionella* growth eliminated the interfering oropharyngeal flora (10). Among these 12 strains, amoebic coculture recovered 2 from samples from patients negative for antigenuria (1 Lp1 Knoxville, 1 Lp8). No significant difference between the performance of *A. castellanii* and that of *A. polyphaga* was observed (the sensitivities of cocultures with these amoeba species were 30% and 36%, respectively [$P = 0.29$; chi-square test]). The type of sample significantly impacted the performance of axenic culture and amoebic coculture (Table 2). The highest sensitivities were obtained with BAL fluid by both methods. No significant difference in the sequence type or MAb subgrouping of the strains isolated by one or the other method was observed.

In this study, we showed that the combination of amoebic coculture with axenic culture enhanced the rate of *Legionella* isolation from respiratory samples and allowed further epidemiological investigations. Amoebic coculture was particularly efficient in isolating *Legionella* spp. from respiratory samples contaminated with oropharyngeal flora and may be systematically applied to such samples.

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