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Adaptation of Amoeba Plate Test To Recover *Legionella* Strains from Clinical Samples

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ABSTRACT The isolation of *Legionella* from respiratory samples is the gold standard for diagnosis of Legionnaires' disease (LD) and enables epidemiological studies and outbreak investigations. The purpose of this work was to adapt and to evaluate the performance of an amoebic coculture procedure (the amoeba plate test [APT]) for the recovery of *Legionella* strains from respiratory samples, in comparison with axenic culture and liquid-based amoebic coculture (LAC). Axenic culture, LAC, and APT were prospectively performed with 133 respiratory samples from patients with LD. The sensitivities and times to results for the three techniques were compared. Using the three techniques, *Legionella* strains were isolated in 46.6% ($n = 62$) of the 133 respiratory samples. The sensitivity of axenic culture was 42.9% ($n = 57$), that of LAC was 30.1% ($n = 40$), and that of APT was 36.1% ($n = 48$). Seven samples were positive by axenic culture only; for those samples, there were <10 colonies in total. Five samples, all sputum samples, were positive by an amoebic procedure only (5/5 samples by APT and 2/5 samples by LAC); all had overgrowth by oropharyngeal flora with axenic culture. The combination of axenic culture with APT yielded a maximal isolation rate (i.e., 46.6%). Overall, the APT significantly reduced the median time for *Legionella* identification to 4 days, compared with 7 days for LAC ($P < 0.0001$). The results of this study support the substitution of LAC by APT, which could be implemented as a second-line technique for culture-negative samples and samples with microbial overgrowth, especially sputum samples. The findings provide a logical basis for further studies in both clinical and environmental settings.

KEYWORDS amoebic coculture, amoeba plate test, Legionnaires' disease, *Legionella* culture, isolation

Legionella species are facultative, intracellular, Gram-negative bacteria of aquatic habitats and water distribution systems; they can survive as free-living bacteria or multiply within amoebae or ciliated protozoa. When humans inhale infectious aerosols, the bacteria can infect and replicate within lung macrophages and cause severe pneumonia, called Legionnaires' disease (LD) (1).

Urinary antigen detection is the first-line diagnostic test but is limited to *Legionella pneumophila* serogroup 1 (Lp1). Using molecular techniques with lower respiratory tract samples can improve diagnosis by detecting other serogroups and species. However, despite a lower sensitivity than that of urinary antigen detection tests or molecular methods, culture of respiratory samples is the gold standard for LD diagnosis because it is specific, and it also enables epidemiological studies and outbreak inves-

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tigations. The sensitivity of culture is notably affected by the *Legionella* inoculum, the use of antibiotics before sampling, the experience of the laboratory team, and the rapid interfering growth of commensal bacteria (2). With regard to the latter point, it was reported that the use of the amoebic coculture method in parallel with culture enhanced the rate of *Legionella* isolation from 42.1% to 47.1%, as amoebae were able to decontaminate the respiratory samples from interfering oropharyngeal flora (3). However, the amoebic coculture procedure described was cumbersome, technically demanding, and very time-consuming.

The amoeba plate test (APT) is an amoebic coculture technique originally described by Miyamoto et al. to study the growth of several *L. pneumophila* strains spotted on agar plates in the presence of *Acanthamoeba culbertsoni* (4). Albers et al. later implemented the APT to analyze the interactions of *L. pneumophila* *icm/dot* mutants with *Acanthamoeba castellanii* (5). In this study, we adapted the APT to clinical settings and evaluated its performance in comparison with routinely used techniques for the recovery of *Legionella* isolates from respiratory samples.

(The results of this study were presented at the 4th ESCMID Study Group for *Legionella* Infections Conference, Amsterdam, Netherlands, 22 to 23 September 2016, and the 36th Réunion Interdisciplinaire de Chimiothérapie Anti-Infectieuse, Paris, France, 12 to 13 December 2016.)

MATERIALS AND METHODS

Patients and samples. This study prospectively included all respiratory specimens that were collected in February 2015 to September 2015 by the National Reference Centre for *Legionella*, as part of routine LD patient management, and were of sufficient volume for axenic culture and amoebic coculture. An LD case was defined as a case with clinical and/or radiological findings consistent with pneumonia and positive urinary antigen results (confirmed LD case) and/or positive *Legionella* PCR results with a respiratory sample (probable LD case). The use of patient data by the National Reference Centre for *Legionella* was approved by the ethics committee of the Hospices Civils de Lyon; written informed consent was not required, in accordance with the regulations in place at the time of the study.

Culture techniques used in this study. All specimens were processed with three culture techniques, i.e., axenic culture, liquid-based amoebic coculture (LAC), and APT (see Fig. S1 in the supplemental material). Axenic culture was performed upon sample arrival at the laboratory, 5 days a week. The samples were liquified using dithiothreitol (Sputasol; Oxoid, Dardilly, France) if necessary. One hundred microliters of each sample was inoculated onto four plates, i.e., buffered charcoal yeast extract (BCYE) (Oxoid); BCYE supplemented with cefamandole, polymyxin B, and anisomycin (BMPA medium) (two plates; Oxoid); and BCYE supplemented with glycine, vancomycin, polymyxin B, anisomycin, bromothymol blue, and bromocresol purple (MWY medium) (Oxoid). The plates were incubated for 10 days at 35°C in an aerobic atmosphere (BCYE and BMPA media) or in a 2.5% CO₂ atmosphere (BMPA and MWY media). A combination of acid decontamination (HCl [pH 2] for 30 min at room temperature) and heat treatment (50°C for 30 min) was performed 24 h later for samples yielding cultures contaminated with oropharyngeal flora (confluent colonies on at least 2 of the 4 plates). The two amoebic coculture techniques were performed weekly, every Monday for LAC and every Friday for the APT; the respiratory samples (liquified or not) were stored at 4°C until coculture. *Acanthamoeba castellanii* (ATCC 30234) organisms were grown in peptone yeast extract-glucose medium at 30°C (6). For LAC, the organisms were suspended in Page's amoebic saline (PAS) buffer, and 10⁶ amoebae were distributed into each well of a six-well tissue culture plate (BD Falcon; Becton Dickinson, Le Pont-de-Claix, France). The APT used in this study was adapted for clinical samples from the test described by Miyamoto et al. and Albers et al. (4, 5); 3.75 × 10⁶ amoebae were suspended in 1.5 ml of PAS buffer and distributed onto a BMPA agar plate. Plates containing amoebae were dried under a laminar flow hood for 1 h before being used. One milliliter of each sample was mixed with sterile distilled water (3 ml) to disrupt the cells, and the sample was centrifuged (220 × *g* for 5 min). The supernatant, which contained bacteria, was removed, divided in two, and centrifuged (9,300 × *g* for 10 min). The first pellet was suspended in 200 μl of PAS buffer and inoculated into a well of the tissue culture plate for LAC. The plate was centrifuged (500 × *g* for 20 min) and then incubated at 30°C. On day 3 after inoculation, 100 μl of the content of the wells was subcultured on BCYE and BMPA media incubated for 10 days at 35°C in a 2.5% CO₂ atmosphere. The plate was incubated for 4 additional days. On day 7 after inoculation, the plate was gently shaken to suspend the amoebae. One hundred microliters of the infected amoebic suspension was then again subcultured on BCYE and BMPA media incubated for 10 days at 35°C in a 2.5% CO₂ atmosphere. A 30-μl volume of PAS buffer was added to the second pellet for APT, and 3 μl of the bacterial suspension was spotted onto the BMPA plate containing the amoeba monolayer. Thus, the minimal number of spots per sample was 10; this number varied according to the volume of the pellet. Plates were incubated for 10 days at 30°C in an aerobic atmosphere. The reference strain Lp1 Paris (CIP 107629T) with *dotA* deleted by a unique insertion of a kanamycin cassette (*dotA::kan*) was constructed by amplifying the *dotA::kan* DNA sequence from the reference strain Lp1 Lens (CIP 108286) with *dotA* deleted (7, 8) and inducing competence for natural transformation in the reference strain Lp1 Paris (9). This strain is unable to infect amoebae and was used

TABLE 1 Comparison of sensitivity of culture and liquid-based amoebic coculture for isolation of *Legionella* from respiratory samples

Culture result	No. of samples ^a		
	LAC positive	LAC negative	Total
Positive	38	19	57
Negative	2	74	76
Total	40	93	133

^a*P* = 0.0005 by McNemar test.

as a control of amoebic viability. Thirty microliters of a bacterial suspension from a 3-day subculture on a BCYE plate (optical density at 600 nm [OD₆₀₀] of 1) was spotted onto a BMPA plate containing an amoeba monolayer during the incubation period and on a BCYE plate without amoebae; the $\Delta dotA$ strain should not form colonies on the APT plate, while it should grow on the BCYE plate. For the three techniques (axenic culture, LAC, and APT), the inoculated plates were read 5 days a week; thus, any plate that could have been detected as positive during the weekend was flagged as positive on Monday.

Identification and typing of *Legionella* colonies. Up to 15 *Legionella* colonies (5 for each technique) per sample were identified from a 1-day subculture on BCYE plates. Identification of *Legionella* colonies was first performed by latex agglutination (Oxoid, Dardilly, France). The Lp1 colonies were typed using sequence-based typing (1 isolate per sample) (10, 11). Identification of non-*pneumophila* *Legionella* strains was performed by *mip* sequencing (12).

Statistical analysis. The sensitivities of the techniques were compared by McNemar tests, using R software (version 3.4.3). The times to results observed with the LAC and APT were compared by a Mann-Whitney test, using GraphPad Prism 6. The impact of sample type on the *Legionella* isolation rate was studied by chi-square test, using R software (version 3.4.3).

RESULTS

Patients and samples. A total of 133 respiratory samples, consisting of 22 bronchoalveolar lavage (BAL) fluid samples, 26 tracheal or bronchial aspirate samples, and 85 sputum samples, were collected from 123 patients, who were 29 to 92 years of age. Among these patients, 108 were confirmed LD cases, while 15 were probable LD cases.

Sensitivity. Using the three techniques, *Legionella* strains were isolated in 46.6% (*n* = 62) of the 133 respiratory samples. Among these 62 *Legionella* culture- and/or coculture-positive samples, 61 were positive for Lp1 and 1 was positive for *Legionella anisa*. No mixed infections by different *Legionella* species or *L. pneumophila* serogroups were diagnosed.

Axenic culture had a sensitivity of 42.9%, which was significantly higher than that of LAC (30.1%; *P* = 0.0005, McNemar test) but not that of APT (36.1%; *P* = 0.07) (Tables 1 and 2; also see Table S1 in the supplemental material). Among the 57 culture-positive samples, 20 samples had <10 contaminating colonies, 13 samples had 10 to 100 contaminating colonies, and 24 samples had >100 contaminating colonies. Six sputum samples had confluent contaminating colonies and were subjected to acid and heat treatment before replating (but not before amoebic procedures were performed); these 6 samples were all negative for *Legionella* by culture (2 persisting overgrown cultures), LAC (5 overgrown cultures), and APT (4 samples for which all spots were contaminated).

Thirty-one samples were found to be positive by all three methods. Nineteen samples were found to be positive by culture and only one amoebic procedure (LAC or APT). Seven samples were found to be positive by axenic culture only, including the sample that was found to be positive for *L. anisa*; for these samples, there were <10

TABLE 2 Comparison of sensitivity of culture and amoeba plate test for isolation of *Legionella* from respiratory samples

Culture result	No. of samples ^a		
	APT positive	APT negative	Total
Positive	43	14	57
Negative	5	71	76
Total	48	85	133

^a*P* = 0.07 by McNemar test.

TABLE 3 Comparison of sensitivity of liquid-based amoebic coculture and amoeba plate test for isolation of *Legionella* from respiratory samples

LAC result	No. of samples ^a		Total
	APT positive	APT negative	
Positive	33	7	40
Negative	15	78	93
Total	48	85	133

^a $P = 0.14$ by McNemar test.

colonies in total. Five samples were found to be positive by an amoebic procedure only; all tested positive by APT, whereas 2 tested positive by LAC (Table S1). However, the comparison of the sensitivities of LAC and APT did not reach statistical significance (30.1% versus 36.1%; $P = 0.14$) (Table 3). Combining an amoebic technique with axenic culture enhanced the isolation rates to 44.4% ($n = 59$) for LAC and 46.6% ($n = 62$) for APT. The APT recovered *Legionella* strains from 5 of the 76 culture-negative samples, i.e., from 6.7% (95% confidence interval [CI], 2.2% to 14.7%) of them (Table 2).

A maximal isolation rate of 65.4% ($n = 17$) was obtained from tracheal or bronchial aspirate samples. This rate decreased to 43.5% ($n = 37$) for sputum samples and 36.4% ($n = 8$) for BAL fluid samples (Table S1). Although a trend was observed, the sample type did not significantly affect the isolation rate ($P = 0.08$, chi-square test).

The 5 additional samples that allowed the isolation of *Legionella* by LAC and/or APT only were all sputum samples (Table S1). In these samples, the ratios between the number of spots resulting in *Legionella* growth and the number of plated spots were 18/18, 14/14, 5/12, 5/10, and 3/10. The numbers of contaminated spots were 0/18, 0/14, 0/12, 1/10, and 5/10 spots, respectively. All 5 samples showed significant contamination by bacteria and/or yeast (>100 contaminating colonies on at least two plates) by axenic culture.

Characteristics of isolated Lp1 strains. Sequence-based typing of the Lp1 strains revealed 26 distinct sequence types (STs). One-half of the isolated strains (29/61 strains) belonged to ST23 ($n = 13$), ST42 ($n = 5$), ST1 ($n = 4$), ST62 ($n = 4$), or ST47 ($n = 3$). No statistical analysis of the ST distribution according to the isolation method could be performed, due to the limited number of strains for each ST and the typing of only 1 isolate per sample, irrespective of the isolation method.

Times to results. Axenic culture led to the identification of *Legionella* strains within 3 to 10 days, with a median time of 5 days (interquartile range [IQR], 4 to 7 days). The APT resulted in identification within a median time of 4 days (IQR, 3 to 5 days). This was significantly shorter than the time to results observed with LAC, which led to identification within 7 to 14 days and for which the median time was 7 days (IQR, 7 to 8 days; $P < 0.0001$, Mann-Whitney test); 39 of the 40 LAC-positive samples were found after the first subculture, before day 11, and only 1 sample was found to be positive after the second plating step on day 14, with a single *Legionella* colony.

DISCUSSION

In this study, we adapted the APT to isolate *Legionella* strains from respiratory samples from LD patients, and we evaluated its performance, in terms of sensitivity and time to results, in comparison with LAC and axenic culture. The respective sensitivities of LAC and APT were not significantly different, but the APT enabled a significant reduction in the median time to *Legionella* identification. The APT notably contributed to the analysis of sputum samples. Regarding the sensitivities of the three evaluated techniques, axenic culture had the highest sensitivity. The only significant difference was found between axenic culture and LAC, and the sensitivities found herein were similar to those reported elsewhere (42.1% for axenic culture and 33.8% for LAC) (3). The sensitivity of APT was between that of axenic culture and that of LAC but was not significantly different from those values, which was possibly related to the limited number of samples included. It also has to be noted that the two amoebic procedures

were performed weekly, whereas axenic culture was performed 5 days a week. Refrigerating the samples before amoeba infections might have impaired *Legionella* viability and reduced the isolation rates for these procedures.

The APT allowed the isolation of *Legionella* strains from 5 additional samples, all of them sputum samples that resulted in an overgrown axenic culture, with >100 colonies on at least one of the three plates containing antimicrobial agents. These results are in line with case reports that demonstrated the ability of *Acanthamoeba* to decontaminate pulmonary or fecal samples and to support *Legionella* growth (6, 13) and a previous study that evaluated the performance of liquid-based amoebic coculture for 240 respiratory samples obtained from LD patients (3). However, *Legionella* was not isolated by any of the amoeba-based techniques tested herein in any of the samples that had confluent colonies by axenic culture and therefore required acid decontamination and heat treatment. This suggests limits of the decontaminating properties of amoebae.

After completion of this study in 2015, our laboratory discontinued the use of LAC and performed both axenic culture and APT for all specimens. In the period from 2015 to 2016, a total of 287 respiratory samples, consisting of 44 BAL fluid samples, 79 tracheal or bronchial aspirate samples, and 164 sputum samples, were processed by both axenic culture and APT; the sensitivities of axenic culture and APT were 29.6% and 24.4%, respectively (see Table S2 in the supplemental material). The APT recovered *Legionella* strains from 15 of the 202 culture-negative samples, i.e., from 7.4% (95% CI, 4.2% to 12.0%) of them.

Regarding the samples collected in 2016, as previously reported herein for those collected in 2015, there were <10 colonies in total for 14 of the 16 samples that were positive by culture only. For 8 of the 10 samples (1 BAL fluid sample, 3 tracheal or bronchial aspirate samples, and 6 sputum samples) that were positive by APT only, the culture was overgrown by >100 colonies on at least one of the three plates containing antimicrobial agent. For those 10 samples, the ratios between the number of spots resulting in *Legionella* growth and the number of applied spots were 1/10, 1/18 (3 samples), 4/16, 5/25, 6/30, 16/17, 10/12, and 12/12. Taken together, these data support the implementation of APT as a second-line technique for respiratory samples with interfering oropharyngeal flora in culture. Interestingly, amoebic coculture has also been reported to contribute to the recovery of *Legionella* strains from environmental samples (14, 15). The APT could be applied to such samples and thus improve the performance of epidemiological investigations and identify new ecological niches for *Legionella* spp.

Mixed lung infections with different *Legionella* species, as well as different *L. pneumophila* serogroups or monoclonal antibody (MAb) subgroups for Lp1, have been reported, mainly by analyzing colonies with different morphologies (16–18). Coscollá et al. diagnosed additional mixed infections by analyzing ST profiles for uncultured respiratory samples from LD patients (19). A limit of the present study is that a maximum of 15 colonies per sample were identified at the species and serogroup level; of the 62 samples yielding *Legionella* isolation, 61 revealed only Lp1 colonies. Moreover, for cost-related reasons, only 1 *L. pneumophila* isolate per sample was subjected to sequence-based typing, irrespective of the isolation method. It would have been relevant to further characterize Lp1 colonies in the samples, in order to possibly detect mixed infections and to determine the ST distribution according to the isolation method, hypothesizing that any ST would be preferentially isolated by amoebic coculture. It has to be noted that not all genera and species of amoebae are permissive for all *Legionella* species and serogroups (15, 20–22). For instance, the species *A. castellanii* supports the growth of several *L. pneumophila* serogroups but is not permissive for all *Legionella* species (15, 22). This is highlighted by the results of the present study; 1 sample was found to be positive for *L. anisa* by axenic culture only, which is understandable because *A. castellanii* has been reported not to be permissive for that *Legionella* species (22). Combining other species of amoebae with *A. castellanii* could constitute an improvement of the APT technique adapted herein.

One major benefit of APT was a more rapid time to *Legionella* identification (4 days),

while LAC required 7 days in this study. The latter technique combines a first step of amoebic infection in liquid medium, followed by two plating steps on days 3 and 7 after inoculation, delaying the last agar plate reading to day 17. It has to be noted that the present study found a weak contribution of the second plating step, with only 1 additional sample being positive on day 14. The adaptation of the APT to clinical samples resulted in a one-step procedure, which avoided a subculture step and simplified the reading scheme (a single agar plate for 10 days). Therefore, the APT was a less time-consuming and less technically demanding procedure that resulted in a shorter delay to *Legionella* isolation. The comparison of times to results for axenic culture (5 days) and APT (4 days) has to be interpreted with caution. Cultures were performed 5 days a week, and it has been shown that >95% of cultures are positive between day 3 and day 5 (23). Thus, times to results for culture have been overestimated due to the lack of weekend reading. In contrast, the APT was performed weekly and the design of the present study supported accurate determination of time to detection for the APT. For this reason, the comparison of times to results by axenic culture and APT was not performed through a statistical analysis.

In conclusion, the APT is easily adapted to isolate *Legionella* strains from respiratory samples and is faster than LAC; its use as a second-line technique after axenic culture could be the most efficient *Legionella* isolation scheme and could provide optimized isolation rates, especially for sputum samples. An interesting future development is the application of the APT to environmental samples.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.01361-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

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