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Incubation of Water Samples Containing Amoebae Improves Detection of Legionellae by the Culture Method

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Some protozoans isolated from aquatic habitats, including domestic water supplies, can support the intracellular replication of autochthonous legionellae in vitro. We studied the effect of incubating water samples containing amoebae on the sensitivity of culture for legionellae. Samples collected during investigations of legionellosis epidemics and shown by conventional culture procedures to contain amoebae, but not legionellae, were incubated at 35°C and replated. Legionellae were recovered from 59 of 144 such samples. Species isolated included *L. pneumophila*, *L. anisa*, *L. bozemanii*, *L. gormanii*, *L. micdadei*, *L. rubrilucens*, *L. sainthelensi*, *L. steigerwaltii*, and an unnamed species. *Acanthamoeba polyphaga*, *Acanthamoeba hatchetti*, a *Rosculus* sp., *Hartmannella vermiformis*, and *Vahlkampfia* spp. were among the autochthonous amoebae identified. Legionellae were recovered by this procedure from only 3 of 63 samples that were negative for amoebae by conventional culture procedures. These results show that water samples negative for legionellae, but positive for amoebae, by standard culture techniques should be incubated and replated to maximize the sensitivity of culture for legionellae.

Legionellae are prevalent in domestic and environmental waters, and legionellosis is transmitted via aerosols generated from these reservoirs (6–8, 16, 18, 21, 23, 32, 34). Disease intervention efforts focus on epidemiologically implicated reservoirs because secondary transmission of legionellosis has not been documented (21). Identification of reservoirs requires the detection of legionellae or their products, and culture is the most specific procedure widely available. The sensitivity of culture, however, is limited by the low densities of legionellae in aquatic environments and the low plating efficiency of standard media for certain strains (6, 9–11, 17, 18, 20, 22, 28, 29, 32).

Several studies have shown that aquatic protozoa, especially amoebae, can provide the intracellular environment required for the replication and persistence of legionellae in situ (1–3, 7, 12, 14, 15, 19, 24–27, 31, 33). Accordingly, incubating water samples containing amoebae could promote *Legionella* proliferation from undetectable to culturable levels. The objective of this study was to determine whether incubating water samples increases the sensitivity of culture for legionellae.

Water samples were collected from domestic distribution systems during investigations of legionellosis epidemics and cultured for legionellae and amoebae. On the basis of primary culture results, samples were selected for incubation at 35°C for 6 weeks and periodically recultured. The methods and data provided in this report are applicable to studies of the aquatic ecology of legionellae and the epidemiology of legionellosis.

MATERIALS AND METHODS

Sample collection. Samples were collected from potable water and cooling tower distribution systems during epidemiologic investigations of legionellosis in three hospitals and a rural community. One-liter water samples were collected from distal plumbing fixtures, storage tanks, and cooling towers in sterile, screw-cap polypropylene bottles. Distal fixtures, primarily shower heads and sink faucets, were also sampled with swabs, and approximately 2 ml of water from the same site was added to the screw-cap sample swab container.

Routine sample treatment. The procedures used to treat and culture water samples are described elsewhere (5). Briefly, 0.1-ml volumes of incubated samples were inoculated onto buffered charcoal yeast extract agar with α-ketoglutarate (BCYEa), BCYEa with antimicrobial drugs, and BCYEa with antimicrobial drugs and glycine. Samples containing rapidly growing, non-*Legionella* microbes were treated with acid, neutralized, and replated. Some samples were concentrated by filtration through a 0.2-μm-pore-size polycarbonate filter in a 47-mm-diameter filter funnel assembly. Approximately 900 ml of water was filtered through an assembly attached to a 1-liter sidearm flask evacuated by an in-house vacuum line. The funnel was disassembled, and the filter was aseptically removed from the support grid, quartered, and transferred to a sterile, 50-ml polypropylene centrifuge tube containing 10 ml of the original sample water. Filtered cells were suspended by being mixed on a Vortex apparatus for 30 s.

Concentrations of legionellae in some samples were calculated from colony counts on single primary isolation plates. Legionellae were identified by direct immunofluorescence staining, enzyme immunoassay using monoclonal and absorbed polyvalent antibodies, or slide agglutination (4, 35).

Amoebae were recovered by a modification (13) of the procedure of Singh (30). *Escherichia coli* cultures distributed over the surface of a nonnutrient agar medium (0.5% NaCl, 1.5% agar) were inoculated with 0.1-ml volumes of water samples. Cultures were incubated for 7 days at 35°C with 2.5% CO_2 and were examined microscopically daily for amoebic cysts and trophozoites. Amoeba cultures from two of the hospitals were stored at 4°C on primary isolation

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TABLE 1. Recovery of legionellae by incubating water samples

Amoebae present[a]	Recovery of legionella[b]	No. of samples[c]
Yes	Yes	59
Yes	No	85
No	Yes	3
No	No	60

[a] Determined by primary culture.
[b] Samples were negative for *Legionella* spp. by conventional procedures.
[c] The total number of samples was 207.

medium and isolated and identified by T. K. Sawyer (Rescon Associates, Inc., Royal Oak, Md.).

Incubation of water samples. Most samples were stored at 4°C until primary results were available, although some samples from past investigations of epidemics were held for up to 6 months. Samples positive for amoebae and negative for legionellae (n = 144), positive for legionellae and negative for amoebae (n = 14), and negative for both organisms (n = 63) were identified by routine culture. Samples representing each category were incubated at 35°C for as long as 6 weeks. Water samples (50 ml) were incubated in sterile, 125-ml screw-cap polypropylene bottles. The filters from water samples that had been concentrated by filtration were also incubated. Swabs were incubated in their respective transport tubes. Samples were cultured for legionellae every 7 to 10 days, using the methods described above. The concentrations of legionellae in 14 samples collected from point-of-use devices of a hospital potable water system were determined by colony counts on triplicate plates of selective media after incubation.

No attempt was made to culture amoebae after incubation of samples. However, the recovery of amoebae during culture of incubated samples on BCYEa media was recorded.

RESULTS

Two hundred seven *Legionella*-negative water samples collected from 71 devices and sites (e.g., cooling towers, plumbing fixtures, and water tanks) were tested. Incubation and subsequent replating resulted in the recovery of *Legionella* spp. from 30% (62 of 207) of the samples (Table 1). These positive samples represented 56% (40 of 71) of all fixtures and sites evaluated. Conventional culture had previously associated legionellae with only 28% (20 of 71) of the sampled devices.

Amoebae were recovered by primary culture in about 70% (144 of 207) of all test samples. *Legionella* spp. were detected by incubation more frequently in samples that contained amoebae (59 of 144, 41%) than in samples that did not (3 of 63, 5%). The rate of culture of legionellae after incubation of amoeba-positive samples varied with the specific water system investigated (30 to 90%). Three incubated samples yielded legionellae although conventional procedures had previously failed to detect amoebae. Amoebae were observed on BCYEa plates of two of these samples during incubation for culture of legionellae. Overall, incubated samples containing amoebae were more likely (Fisher's exact test, $P = 1.9 \times 10^{-8}$; odds ratio = 13.9; 95% confidence interval, 3.9 to 37.3) to yield legionellae than those that did not.

The concentrations of legionellae (CFU per milliliter) did not increase after incubation of 14 samples from which *Legionella* spp., but not amoebae, were initially recovered. In fact, *Legionella* concentrations decreased at least 10-fold in 13 of these samples during the incubation period.

Various *Legionella* spp. were isolated after incubation of samples that were negative by conventional procedures, including *L. anisa*, *L. bozemanii*, *L. gormanii*, *L. micdadei*, *L. pneumophila* (serogroups 1, 2, 3, 5, 6, 7, and 10), *L. rubrilucens*, *L. sainthelensi*, a *Legionella* sp. (an unidentified species phenotypically similar to strains designated 1224JD that are under evaluation), and *L. steigerwaltii*. Amoebae identified in samples included *Acanthamoeba polyphaga*, *Acanthamoeba hatchetti*, *Hartmannella vermiformis*, a *Rosculus* sp., and *Vahlkampfia* spp. *H. vermiformis* was isolated from the greatest number of samples, but no single species was found in all samples, devices, or water systems.

DISCUSSION

Epidemiologic and bacteriologic studies show that *Legionella* spp. inhabit a multitude of aquatic environments and can survive in aerosols created from these waters (6, 8, 16, 18, 21, 32, 34). Legionellosis can occur if these aerosols are inhaled by a susceptible host; however, no secondary transmission of legionellosis has been documented (21). Consequently, control and prevention of this disease are contingent on detecting aquatic reservoirs of legionellae. The culture of water samples for *Legionella* spp. is the most specific detection method widely used, but it may not be highly sensitive. Rowbotham (25, 26) proposed the use of amoebae to improve recovery of legionellae from water systems and later cultured *L. pneumophila* after adding *A. polyphaga* to clinical specimens. Our study showed that incubating water samples containing autochthonous amoebae markedly improved the sensitivity of culture for legionellae.

Sample incubation enabled the recovery of epidemic *Legionella* strains and identified reservoirs that were not recognized during epidemiologic investigations with routine procedures. Nonepidemic strains and the more fastidious legionellae such as *L. micdadei* were also isolated by incubation, indicating that this technique is not species restricted.

Legionella spp. were more likely to be isolated from samples that contained amoebae than from those that did not. *Legionella* concentrations decreased in incubated samples without amoebae. These observations suggest that legionellae increased in concentrations to culturable levels by replicating in amoebae rather than by converting from nonreplicating to replicating forms (20). Alternatively, conversion may occur only when amoebae are present. In either case, in situ persistence, as well as multiplication of legionellae, may be related to indigenous amoebae.

The presence of *H. vermiformis*, the most common amoebic isolate in this study, was previously correlated with the recovery of an epidemic strain of *L. pneumophila* from potable water sites (7). However, several *Legionella* spp. were isolated after incubation of samples containing indigenous amoebae exclusive of *H. vermiformis*. This finding suggests that replication was not dependent on a single genus of amoebae and that aquatic legionellae were adapted to replication within autochthonous amoebae. Given the common occurrence of amoebae in water systems, incubation seems a simpler and more efficient enrichment strategy than the addition of exogenous amoebae (26). The latter technique, however, may be useful for enriching concentrations

of *Legionella* spp. in samples that do not harbor indigenous amoebae.

Experimental evidence shows that the accurate measurement of *Legionella* concentrations is unlikely if samples are maintained at elevated temperatures for extended periods. Thus, studies that report CFU of legionellae in water samples should describe storage and transport conditions and procedures. If accurate assessments of in situ concentrations of legionellae are needed, samples should be refrigerated as recommended elsewhere (5).

Incubating water samples increased the sensitivity of culture for *Legionella* spp. in this study. No additional materials or equipment beyond those used for conventional culture were needed. The primary culture of amoebae could be used as an indicator for sample incubation and reculturing for legionellae. If amoebae are not routinely cultured, all samples that are negative for legionellae by conventional procedures should be incubated and replated. The incubation technique should be used in investigations of epidemics and studies of the aquatic ecology of *Legionella* spp.

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