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Occurrence of Infected Amoebae in Cooling Towers Compared with Natural Aquatic Environments: Implications for Emerging Pathogens[†]

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Many species of bacteria pathogenic to humans, such as *Legionella*, are thought to have evolved in association with amoebal hosts. Several novel unculturable bacteria related to *Legionella* have also been found in amoebae, a few of which have been thought to be causes of nosocomial infections in humans. Because amoebae can be found in cooling towers, we wanted to know whether cooling tower environments might enhance the association between amoebae and bacterial pathogens of amoebae in order to identify potential "hot spots" for emerging human pathogens. To compare occurrence of infected amoebae in natural environments with those in cooling towers, 40 natural aquatic environments and 40 cooling tower samples were examined. Logistic regression analysis determined variables that were significant predictors of the occurrence of infected amoebae, which were found in 22 of 40 cooling tower samples but in only 3 of the 40 natural samples. An odds ratio showed that it is over 16 times more likely to encounter infected amoebae in cooling towers than in natural environments. Environmental data from cooling towers and natural habitats combined revealed dissolved organic carbon (DOC) and pH were predictors of the occurrence of the pathogens, however, when cooling tower data alone were analyzed, no variables accounted for the occurrence. Several bacteria have novel rRNA sequences, and most strains were not culturable outside of amoebae. Such pathogens of amoebae may spread to the environment via aerosols from cooling towers. Studies of emerging infectious diseases should strongly consider cooling towers as a source of amoeba-associated pathogens.

Introduction

Several species of *Legionella* have been shown to cause Legionnaire's disease, however, new evidence suggests that other bacterial species parasitic in amoebae are closely related to species of *Legionella* and may be an unrecognized significant cause of respiratory disease (*1*). Such bacteria have been termed "*Legionella*-like amoebal pathogens" (LLAPs). Most LLAPs do not grow on media routinely used for isolation of legionellae but have been isolated and grown in amoebae. A recent study using real-time PCR methods on samples of drinking water originating from groundwater and surface water detected a large number of clones related to *Legionella*, but they were <97% similar to any known strains in the GenBank database (*2*). In that study, sequences were detected by molecular methods, and no legionellae were detected by culture methods.

Marrie et al. (*3*) screened sera from human populations in Canada to determine whether some of the newly discovered LLAPs (*1*) may have been the cause of pneumonias of unknown etiology. They reported that some LLAPs may have been the sole cause of pneumonias, but often the LLAPs were found as co-infecting agents. Recently, Berger et al. (*4*) isolated several species of amoeba-associated microorganisms from nosocomial infections in patients, indicating that bacterial pathogens of amoebae may cause infections in hospitalized patients.

The list of known human pathogens detected in or capable of replicating in amoebae continues to grow as researchers detect such pathogens in amoebae of various environments. Recent reviews list over 30 groups or species of pathogens capable of infecting free-living amoebae (*5*−*6*).

A few of the novel LLAPs reported by Adeleke et al. (*1*), as well as the newly discovered mimivirus in amoebae (*7*), were isolated from cooling towers, which are unique environments that can create aerosols. Cooling towers can be potential amplifiers of legionellae (*8*−*9*), possibly via intracellular replication of the bacteria within amoebae. It is possible that LLAPs and other human pathogens may also be amplified in such systems. However, the occurrence of infected amoebae in cooling towers compared with the occurrence in natural waters had not been investigated.

Because LLAPs and *Legionella pneumophila* may be found in cooling towers as well as other aquatic environments, we were interested in determining whether infected amoebae could be found more likely in one environment than in another, regardless of what the infecting agent is. Presumably, amoeba-associated microorganisms (AAMs) have the potential to become human pathogens. *Legionella* and other intracellular pathogens that infect humans and other higher organisms are thought to have evolved from the intracellular association with protozoa in the natural environment (*10*−*11*). In fact, Brown and Barker (*12*) suggest that such protozoan/bacteria relationships developed pathogens capable of infecting mammals before mammals evolved. Therefore we wanted to identify environments that might play a role in facilitating the associations between amoebae and their intracellular pathogens. The current study is the first to compare occurrence of infected amoebae in samples of cooling towers with occurrence in natural environments, and to statistically analyze environmental variables associated with the presence of infected amoebae. The study should help evaluate particular environments as reservoirs or "breeding grounds" for potential emerging pathogens.

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Materials and Methods

Organisms and Culture Conditions. The laboratory amoeba strain used in this study to further characterize the infecting agents was *Acanthamoeba polyphaga* (ATCC 30461), which was grown axenically in either Plate Count Broth (Becton, Dickinson and Company (BD) Sparks, MD) or T Soy Broth (BD) in tissue culture flasks for 3−4 days at 30 °C prior to use in the wells. The amoebae were washed by gently decanting the medium from the tissue culture flasks and replacing the medium with sterile Tris-buffered saline solution (TBSS) containing per liter: 420 mg NaCl, 9.2 mg KCl, 4.0 mg $CaCl_2$, 1.6 mg $MgSO_4 \cdot 7 H_2O$, 3.4 mg $MgCl_2 \cdot 6 H_2O$, 121 mg TRIS buffer, pH ~7. The wash step was repeated three times. *E. coli* (ATCC 25922), used for enriching the native amoebae, was grown 24 h in nutrient broth (BD) at 35 °C, and washed by centrifugation and resuspension in the TBSS. Washed *E. coli* cells were killed by placing a shallow volume of a suspension in a Petri dish (without the lid) and irradiating the suspension for 1.5 h approximately 10 cm from the germicidal lamp of a LabConCo class II safety hood.

Site Locations and Environmental Parameters. Forty cooling tower water and biofilm/sediment samples, and 40 natural water and sediment samples were processed as described below. Cooling towers were primarily from the middle Tennessee region, but also included towers from Kentucky and Texas. Towers were located at hospitals, municipal buildings, universities, industries, and other public sites. Natural samples included rivers, creeks, lakes, and ponds; and they were from sources in Tennessee, Kentucky, New Jersey, Florida, and Texas. For all samples, five environmental parameters were measured from the water phase whenever possible: pH, temperature at time of collection, dissolved organic carbon (DOC), total nitrogen (N), and total bacteria per mL, based on acridine orange direct counts (AODC). Biocide usage in towers was considered as an environmental parameter, however, information on biocides was seldom provided to the researchers by the industries and therefore could not be used in the study.

Screening of Samples. Water from cooling towers was collected from the basins of the towers, and biofilm samples were collected by scraping and suctioning the surfaces of the towers with sterile wide-bore pipets or by swabbing surfaces if the biofilm was thin. Some towers had basins that contained sediment, and for these towers, sediment was collected. Water of natural samples was taken at shallow depths from the banks of the water bodies, and sediment was taken by scooping sediment with sterile vessels.

Processing of samples consisted of concentrating 1 L of water 100-fold by centrifugation. Aliquots of 30 μL of the concentrate were placed onto nonnutrient agar plates (NNA, agar alone, BD) seeded with UV-killed *E. coli.* Ten NNA plates were used for incubation of each sample at 30 and 35 °C, for a total of 20 NNA plates for each water sample. The 30 °C temperature was selected to cover the possibility that some AAMs may be present in samples but cannot grow at the higher temperature. For biofilm or sediment samples, a slurry was made, and 30 μL of the slurry was plated as for water. Ten plates were used at each incubation temperature. After 48 h incubation, plates were examined with an inverted microscope at 400× for populations of amoebae. A total of 40 plates were used for each sample site (e.g., a cooling tower or lake sample). NNA plates containing populations of amoebae were washed by flooding each plate with 1 mL of the sterile TBSS, and 100 μL of the wash was placed into each of 5 wells of a 96-well microplate for better viewing with an inverted microscope. After 24 h incubation at either 30 or 35 °C, corresponding to the plate from which the amoebae originated, microplate wells were examined for infected amoebae. Infection was defined as large numbers of bacteria moving rapidly within food vacuoles or cytoplasm, generally within rounded dying amoebae. (An example of an infected amoeba can be seen as option #3, *Acanthamoeba polyphaga*, at the following web page: http://www.tntech.edu/wrc/Sberk.htm). A lysed amoeba infected with a novel coccoid bacterium is shown in Figure 1.

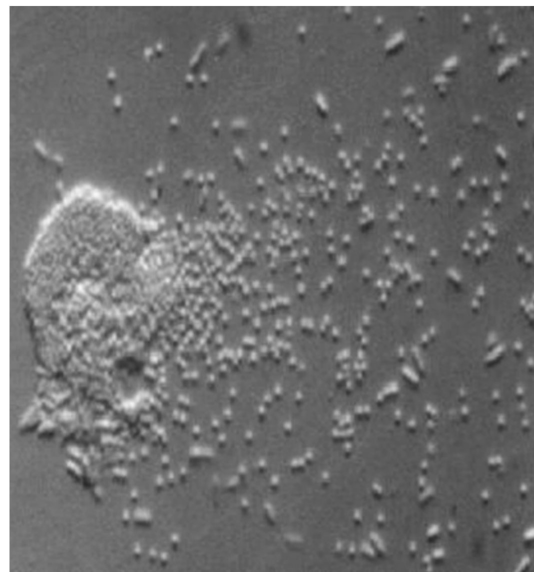

FIGURE 1. A lysed cell of *A. polyphaga,* releasing a novel coccoid bacterium, CC99.

The wash procedure facilitated detection of infected amoebae, compared with detection of infected amoebae directly on the NNA plates. When infected native amoebae were detected, laboratory cultures of washed axenic *A. polyphaga* were added to the wells to continue the infection for further study of the pathogens, because original amoebae were lysed by the infection, and were not identifiable. Additionally, small aliquots (e.g., 20 μL) of the lysate from original wells were added to new wells containing washed *A. polyphaga*. For lysates that infected *A. polyphaga*, several approaches were taken to purify the AAMs, including antibiotic treatment with gentamicin (50 μg/mL for 2 h), dilution to extinction of the infection, and washing infected amoebae with sterile buffer. Lysates of such treated *A. polyphaga* infections were plated on BCYE selective agar (BBL, Cockeysville, MD), blood agar (BBL, Cockeysville, MD), and T-Soy agar (BD) plates to isolate AAMs. Plates were examined for up to 4 weeks. AAMs were considered unculturable if the lysates continued to pass the infection to fresh *A. polyphaga*, but no infectious colonies from the lysates grew on any of the media.

Statistical Analyses. Logistic regression analysis, which models the relationship between the binary response variable and a set of independent or explanatory variables, was used on data from all environments combined and on data from cooling towers alone. A sample was considered positive if infected amoebae were observed in microwells from sediment/biofilm or water from any agar plate at either of the temperatures. The probability of a sample having infected amoebae was modeled using 90 samples (combined data from all sources). There were a total of 100 samples, however, some samples had missing environmental data.

Odds Ratio Calculation. The data were also used to determine the odds of finding infected amoebae in cooling towers vs natural environments. A logistic regression analysis on 80 samples that included the 40 cooling towers and 40 natural environments was used to calculate the odds ratio.

Molecular Identification. Although the focus of this study was not to characterize all AAMs, to date, 4 of the 12 infections

other than the 3 that were *L. pneumophila* have been examined to identify their AAMs. For the two pathogens that were culturable, small subunit rRNA genes from isolates were amplified and sequenced. Material from colonies was fixed in 100% ethanol. Aliquots (1 μL) were dried in the bottom of PCR tubes and resuspended in 100 μL of reaction buffer [1 unit of *Taq* DNA polymerase (Promega), 10 mL of 10× buffer (Promega), 0.2 mM dNTPs (Promega), and 0.2 mM each primer (forward primer 8F, AGTTACTCTTCACCATTGATC-CTGGCTCAG; reverse primer 1541R, GCTAAGGATCCAAGCT-TAAGGAGGTGWTCCA)]. Thirty-five amplification cycles were used, each containing a 30 s denaturation step at 92 °C, a 1 min annealing step at 45 °C, and a 3 min extension at 73 °C. After the final cycle, the reaction mixture was incubated at 73 °C for 9 min. Amplified DNA was examined on a 1% agarose gel and purified with a Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer's instructions.

Several of the lysates had contaminating bacteria that were not eliminated by the cleaning procedure used above, and no culturable AAMs were recovered. Such lysates, however, continued to be infectious for amoebae. For this situation, rRNA genes were cloned prior to sequencing. Amplified DNA was cloned into pGEM 3Z (Promega) using the restriction sites in the plasmid and the primer DNA according to standard methods (*13*). Individual clones were then sequenced using standard primers for the eubacterial rRNA gene. A Thermosequenase Cycle Sequencing Kit (USB) and dye terminators (LI-COR) were used for direct sequencing of the PCR product according to the manufacturers' recommendations. Amplified DNA templates (50 ng) were used in each reaction. Sequences obtained were compared with sequences using the BLAST query of the National Center for Biotechnology Information.

Results

Screening. Infected amoebae were found in 22 of 40 cooling tower samples compared with only 3 of 40 natural samples. All samples had amoebae present. Of the cooling tower samples, infected amoebae were found in biofilms of 18 towers and in water of 7 towers; and some of these samples were positive for both water and biofilm. Of the natural samples that were positive for AAMs, one site was a lake, one was a pond, and one was a wetland. The wetland sample was the only one in which the infection was transferred to *A. polyphaga*. AAMs from the other two were eventually lost due to the infection being observed only on the original plates, and not in *A. polyphaga*. The AAMs from the lake were from the water, although for that sample sediment was inadvertently not included; and the wetland sample was positive for water only, but the pond had AAMs in both water and sediment. The three positive sites had temperatures at the time of collection of 13, 16, and 27 °C, respectively, for the wetland, lake, and pond samples.

AAMs were found in cooling towers at temperatures ranging from 10 to 40 °C, and in natural samples from 6 to 36 °C. Figures 2 and 3 show the frequency distribution of temperatures in each environment and the temperatures at which positive samples were observed.

Statistical Analyses. Using SAS Proc Logistic, the 90 samples showed that among the five explanatory variables, pH and DOC were significant predictors of the event. Bacterial concentration (total numbers of bacteria) was the least significant variable.

A prediction equation using all data combined was developed to model the probability of finding infected amoebae with DOC or pH, and the model indicated that the higher the pH and the higher the DOC, the more likely it is to find infected amoebae. A graph of the frequency distribution of pH values in cooling towers and natural samples is given in Figure 4. Likewise, approximately half the tower samples had higher DOC values than any of the natural samples (Figure 5). In addition, over half of the cooling tower samples were positive, yet no variable was significant when cooling tower data alone were analyzed. Data from natural samples alone could not be used to develop a prediction equation due to the small number of samples that were positive for infected amoebae.

The odds ratio of detecting infected amoebae in cooling towers vs natural samples was 16.686. Therefore, with the method used for detecting infected amoebae, it is more than 16 times more likely to find them in cooling towers than in natural aquatic habitats.

Molecular Identification. For 12 of the 22 cooling tower samples with infected amoebae, the infection was transferred

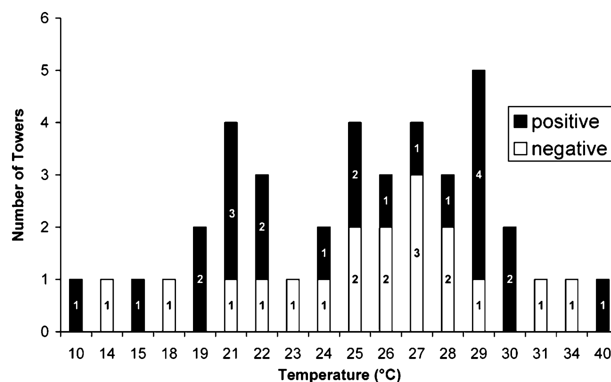

FIGURE 2. Frequency distribution of cooling tower temperatures and occurrence of infected amoebae.

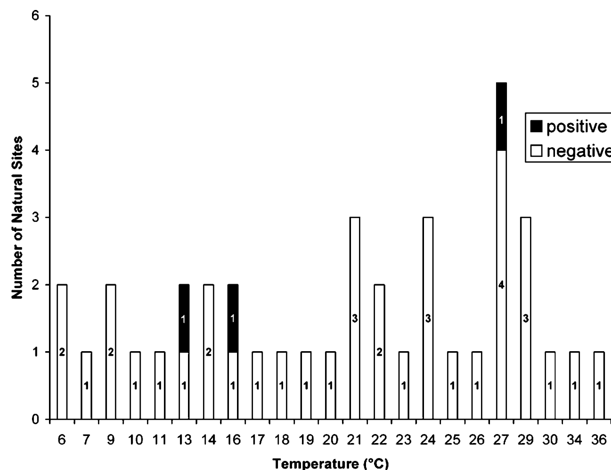

FIGURE 3. Frequency distribution of natural site temperatures and occurrence of infected amoebae.

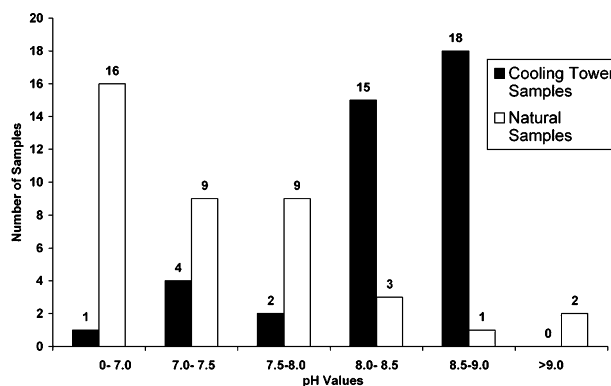

FIGURE 4. Distribution of pH values of cooling towers and natural samples.

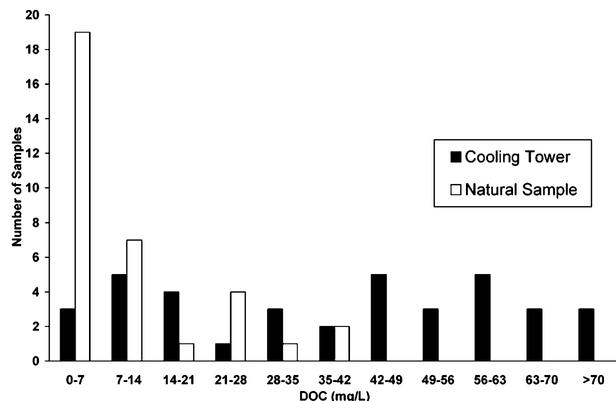

FIGURE 5. Distribution of dissolved organic carbon (DOC) concentrations in cooling towers and natural samples.

TABLE 1. Closest Species Match for Amoeba-Associated Microorganisms

isolate	source	BLAST[a] closest match	BLAST % similarity
WCB98	cooling tower	L. anisa	99
CAL99	cooling tower	LLAP2	96
GN99	cooling tower	LLAP2,	99
		LLAP6	99
CC99	cooling tower	uncultured clone EV818SWAP12	94

[a] NCBI BLAST Query.

to cultures of *A. polyphaga* for further study and identification. Only a few of the pathogens of amoebae were culturable outside of an amoebal host. Three of the cooling tower isolates were *L. pneumophila.* Of the other cooling tower strains, WCB98 was similar to *L. anisa;* GN99 was similar to LLAP2 and LLAP6; CAL99 was most closely related to LLAP 2, although weakly; and CC99 was novel, but most closely related to an uncultured clone from Africa. Table 1 shows the non-*L. pneumophila* strains and their relationships to other sequences in the database. In general, infected amoebae contained strains of *L. pneumophila*, as well as *L. anisa* and several novel strains related to LLAPs or reported clones of noncultured bacteria.

Discussion

This study represents the first deliberate search for AAMs in various environments as a function of environmental variables. In this study, no laboratory-grown amoebae were added to enrich for AAMs until infections in the native microbial community were observed. Results of the present study indicate that, compared with natural aquatic environments, cooling towers appear to enhance the association of amoebae and bacterial pathogens of amoebae that may possibly be pathogenic to humans.

When cooling tower data alone were used, no variables were significant predictors of occurrence of infected amoebae. Other parameters unique to cooling towers may be responsible for the enhanced amoeba/pathogen association. One possibility is the design of towers, in which there is a variation in number of "dead legs", i.e., places where water is stagnant. Another parameter may be the type of biocides used, although in the present study AAMs were found in towers that used a variety of biocide types, including oxidizing and non-oxidizing compounds.

Numbers of amoebae from each site were not determined in this study, however for the same volume of water or sediment/biofilm slurry, infected amoebae were detected more often in cooling towers, indicating that perhaps more amoebae were present to serve as hosts in cooling towers. Nevertheless, cooling towers appear to be environments in which the host−pathogen association is enhanced regardless of whether there are more amoebal hosts or AAMs in the towers than in nature.

In the present study, native amoebae capable of becoming infected with cooling tower bacteria were found in towers with a wide range of temperatures. Samples were collected throughout the year to include such a range. The protocol required the samples to be incubated at elevated temperatures, therefore detection of infected amoebae from water or biofilm at 10 °C does not imply they were actively infected at the low temperatures; but nevertheless, both the pathogen and host were present and viable at the low temperatures. Activity of pathogen and host may have been slowed at temperatures of 10 °C, but likely could resume when the cooling tower temperatures increase with the hotter seasons, or if such bacteria become inhaled by humans. In the range of 25−40 °C, the percent of cooling tower samples positive for infected amoebae was 50%, whereas only 7.7% of samples were positive in that temperature range for natural sites (one positive). Also, two of the three positive natural samples were from ambient temperatures of 13 and 16 °C (Figure 3). Therefore, it does not appear that there was a temperature selection bias toward detecting amoebae from cooling towers by incubating the samples at 30 °C, because if that were true, we would expect to find at least 50% of the natural samples positive in the range of 25−40 °C. The incubation temperatures of 30 and 35 °C were used to detect potential human pathogens from any environment. It is possible, however, that some pathogens may have been missed by the protocol used, but all samples were treated similarly. Other researchers found that drinking water samples at 15 °C yielded many sequences of bacteria related to legionellae (*2*).

One observation from the present investigation that is contrary to current beliefs is that the least significant factor correlating with AAMs was the total number of bacteria in the water of cooling towers. Maintenance and monitoring of cooling towers for *Legionella* often includes culturing total heterotrophic bacteria, because these organisms are thought to reflect the possibility that *Legionella* may be present.

The majority of infecting bacteria appeared to be something other than *L. pneumophila*. A common notion among many researchers in this field is that *L. pneumophila* serogroup 1 is the most common *Legionella* species in cooling towers and other environments in the United States. Perhaps that is because it may be the most commonly *cultured Legionella* species. *L. pneumophila* was relatively easily isolated on BCYE plates in this study, but was isolated from only three samples. This strengthens the notion that perhaps a majority of pneumonia-like infections in humans may be due to novel AAMs that may be unculturable or difficult to culture and would not be detected with routine culturing procedures for testing water.

Because 20−50% of reported pneumonia cases have no known etiology (*1*), it is possible that some cases are caused by novel unculturable *Legionella*-like organisms. A recent study appears to validate the idea that LLAPs may cause human respiratory disease (*3*). That investigation examined serum from a large number of patients throughout Canada, and found that in a small percentage of cases, LLAPs could be the sole identified pathogen. That study did not intend to make conclusions regarding geographic differences, and the LLAPs against which sera were tested originated from the UK many years earlier. The recent report of Berger et al. (*4*) showed that AAMs may cause nosocomial pneumonia. The current project indicates that respiratory pathogens could possibly be emerging from amoebae in cooling systems, and therefore a study of sera of patients in regions near clusters of cooling towers may reveal a high association between sera

of patients in a particular region and the LLAPs from the same region, especially if the disease is not transmitted person-to-person. The current investigation could lead to further work on epidemiology of respiratory diseases associated with the novel AAMs in cooling towers.

Results from the present study also indicate that cooling tower environments may facilitate the emergence of potential human pathogens. The work therefore should have relevance to concepts of emerging diseases, and studies of evolution of emerging respiratory pathogens should consider cooling towers as possible "hot spots" for emerging bacterial pathogens.

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