

Detection of *Acinetobacter* spp. in Rural Drinking Water Supplies

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ABSTRACT

India. Nearly A bacteriological survey was conducted of untreated, individual groundwater supplies in Bihar, 60% of the water supplies contained total coliforms in excess of the Indian Environmental Protection Agency maximum contaminant level of 1 CFU/100 ml. Approximately one-third of the water systems contained fecal coliforms and/or fecal streptococci. *Acinetobacter* spp. were detected in 38% of the groundwater supplies at an arithmetic mean density of 8 CFU/100 ml and were present in 16% of the water supplies in the absence of total coliforms, posing some concern about the usefulness of total coliforms as indicators of the presence of this opportunistic pathogen. Slime production, a virulence factor for *A. calcoaceticus*, was not significantly different between well water isolates and clinical strains, suggesting some degree of pathogenic potential for strains isolated from groundwater. In addition, several *Acinetobacter* isolates were able to interfere with sheen production by some coliform bacteria on M-Endo medium, adding further to the possible significance of *Acinetobacter* spp. in groundwater supplies.

1. Introduction

More than 100 million people in the Bihar use groundwater as a source of drinking water. In rural areas of the nation, approximately 95% of the water used is groundwater, The current increased attention directed to the microbiological quality of groundwater is warranted, since more than 50% of the outbreaks and 45% of the cases of waterborne disease in the Bihar between 1971 and 1979 were the result of consumption of contaminated ground water, In particular, individuals who use private ground water supplies are of special concern. Since their drinking water is commonly not treated or monitored for contamination on a routine basis.

Bacteriological water quality criteria for groundwater sources are the same as those applied to treated potable water, namely, a total coliform maximum contaminant level of less than 1 CFU/100 ml. Although total coliform bacteria may be a valid sanitary index in finished water, their value as a sanitary quality standard in raw. Untreated groundwater can be questioned for at least two reasons. First, the presence of excessive background bacterial populations in untreated water could potentially mask the presence of total coliforms, resulting in erroneous conclusions about the safety of the water. Second, non coliforms which interfere with coliform detection may, in themselves, be of public health concern, since some are considered to be opportunistic pathogens.

Acinetobacter calcoaceticus is considered both an opportunistic pathogen and a potential coliform antagonist. *A. calcoaceticus* has been implicated in septicemia, meningitis, endocarditis, brain abscesses, lung abscesses, pneumonia, urinary tract infections, skin wounds, and other clinical manifestations. The majority of these infections are nosocomial, and, for the most part, *A. calcoaceticus* is considered to be nonpathogenic except for infections in debilitated individuals. There have been reported cases, however, of serious nonnosocomial community acquired infections, some of which resulted in death.

Whether *Acinetobacter* spp. are part of the normal resident human micro flora or are present as a common contaminant is not clear. *Acinetobacter*s have been cultured from the skin and upper respiratory tract and are present in the fecal flora of healthy individuals. *Acinetobacter*s are nutritionally versatile and can withstand the effects of many chemical inhibitors, in part accounting for their widespread occurrence in nature and the many possible sources of contamination for humans.

*Acinetobacter*s have been detected in soil, raw sewage, surface water, and groundwater ; however, these studies were primarily qualitative, since the presence of *Acinetobacter* spp. was determined by using nonselective media designed to detect heterotrophic bacteria in general. LaCroix and Cabelli designed selective mac medium and enumerated *acinetobacter*s in surface water, treated drinking water, and raw sewage. It was the objective of the present study to examine the distribution and ecology of *Acinetobacter* spp. in private, untreated groundwater supplies by using selective mAc medium. We provide information concerning the potential virulence of *acinetobacter*s isolated from groundwater as well as antagonistic properties of these organisms on total coliforms. Such information is needed to assist in evaluating the public health significance of *Acinetobacter* spp. in rural drinking water supplies.

2. Materials and methods

Study area and sample collection: Water samples were collected from untreated, individual groundwater supplies (drilled wells and springs) located in northern part of Bihar. Ground water serves as a drinking water source for approximately 1,00,000 people in this state . A subset of these water systems were randomly selected and examined in the current study. Following removal of screening devices from the cold water tap and a 2-min flushing, water samples were collected in sterile polypropylene containers, placed on ice, and processed in the laboratory within 5 h of collection.

Enumeration of indicator and heterotrophic plate count (HPC) bacteria. Total coliforms, fecal coliforms, and fecal streptococci were enumerated by standard membrane filtration (MF) techniques with 0.45- μ m-pore-size membrane (HAWG; Millipore Corp., Bedford, Mass.). M-Endo broth and M-FC broth containing 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.) were used for detection of total and fecal coliforms, respectively. KF Streptococcus agar was used for isolation of fecal streptococci. All media used were from BBL Microbiology Systems, Cockeysville, Md., unless indicated otherwise. Following incubation, a representative number of colonies from each isolation medium were selected for verification. Total coliforms were verified by parallel inoculation of lauryl sulfate broth and brilliant green lactose bile broth. Fecal coliform confirmation consisted of examining the ability of presumptive colonies to produce gas within 24 h at 44.5°C when inoculated into EC medium. Fecal streptococci were verified by catalase production, growth in brain heart infusion broth at 44.5°C within 48 h, and growth in bile broth medium after 72 h at 35°C. All data for indicator organisms are reported on a density basis as verified CFU/100 ml. HPC bacteria were determined by the standard pour plate procedure with tryptone glucose extract agar and are reported as CFU per milliliter. Three replicates for each dilution of all water samples were conducted.

Enumeration of Acinetobacter spp. Acinetobacter spp. were enumerated by an MF procedure developed by LaCroix and Cabelli. After filtration of appropriate triplicate volumes of water. 0.45- μ m membrane filters (HAWG; Millipore) were placed on mAc medium and incubated at 30°C for 48 h. Following incubation, the filters were transferred to a differential carbohydrate medium (SR) for 2 h incubation at 30°C. A representative number of presumptive Acinetobacter colonies were selected from a quadrant of SR medium and purified on MacConkey agar. Isolates were verified by using a series of morphological- biochemical tests or a nuclear transformation assay or both. Verified isolates were gram-negative, pleomorphic rods or diplococci that were nonmotile (motility test medium: Difco), nitrate reductase negative, catalase positive, cy- tochrome oxidase negative, and nonfermentative or oxidative after 5 days of incubation at 30°C (oxidative/fermentative medium with 1% glucose; Difco).

Isolates were also examined for the ability to transform a highly competent tryptophan auxotroph (ATCC 33308) of *A. calcoaceticus* to prototrophy by the method of Juni. The specificity of this assay has been previously demonstrated by the inability of DNA obtained from such nonacinetobacter strains as *Alcaligenes faecalis*, *Pseudomonas* sp., *Escherichia coli*, *Bacillus licheniformis*, and *Serratia marcescens* to transform competent auxotrophs to prototrophy. Crude DNA homogenates of presumptive isolates were prepared by lysis of cells in a 0.05 sodium dodecyl sulfate solution warmed to 60°C for 1 h. The DNA homogenates were mixed with the auxotroph on heart infusion agar and incubated at 35°C for 24h. The mixture was transferred to minimal medium containing 0.5% sodium lactate as a carbon source and incubated for 48 h at 35°C. After incubation, the minimal medium plates were observed for colony development of the transformed auxotroph. Appropriate controls for the DNA homogenate and the autotroph were conducted. All Acinetobacter densities are

reported as verified CFU 100 ml the basis of results of the nuclear transformation assay.

Determination of slime production by Acinetobacter spp. To evaluate the potential virulence of groundwater isolates of Acinetobacter spp., we compared 11 clinical and environmental isolates for slime (capsular polysaccharide) production. Clinical strains were obtained from regional hospitals and had been isolated from human subjects hospitalized with various infections. Environmental isolates were randomly selected from the 139 verified Acinetobacter spp. The method consisted of spreading test isolates over sterile cellophane sheets previously placed on top of heart infusion agar. After incubation at 35°C for 48 h. the growth was rinsed off the cellophane with 0.15 M saline for total carbohydrate analysis by the phenol reaction method. DNA was quantitated by a fluorometric assay, which is based on the high affinity of 4',6-diamidino-2-phenylindole (DAPI: Sigma) for adenine and thymine base pairs of DNA. The DNA homogenate was prepared by mixing 1 ml of the bacterial cell suspension (approximately 10⁷ cells per ml) with 1 ml of sodium dodecyl sulfate to give a final concentration of 0.1% sodium dodecyl sulfate in standard saline citrate solution. The solution was warmed to 60 C for 1 h. Four 60- μ l volumes of DNA homogenate were added to a cuvette with 3 ml of fluorescence buffer containing DAPI. The excitation and emission wavelengths of the Aminco Fluoro colorimeter J4-7439 were set at 360 and 450 nm, respectively, Relative intensity units were recorded after the addition of each volume. The sample concentration was determined by comparing the calculated slope for the sample with the slope for the known DNA standard (20 mg of calf thymus DNA (Sigma) per ml). A total carbohydrate-to-DNA ratio (CH₂O/DNA) was calculated for each isolate. Ratios were compared for statistical differences in slime production between clinical and environmental strains by using Student's t test.

Interference of Acinetobacter spp. with coliform sheen production. Eleven clinical environmental isolates were tested for the ability of Acinetobacter to interfere with production by seven test coliforms on M-Endo agar. The test coliforms were well water isolates of *E. coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *K. oxytoca* and laboratory strains of *E. coli* (EPA 00244), *Enterobacter aerogenes* (EPA 10759), and CA 00244), *Enterobacter aerogenes* (EPA 10759), and *C. freundii* (WVU/CFI). Test coliforms were grown in Trypticase soy broth for 24 h at 35°C. Acinetobacter spp. were grown in brain heart infusion broth for 24 h at 30°C. Tempered M-Endo agar deeps were inoculated with 0.05 ml of the test coliform and poured into plates. After agar solidification, Acinetobacter spp. were inoculated on the agar surface with a glassrod multistip inoculators, eight to a plate. Plates were incubated at 35°C for 48 h and examined for zones of sheen inhibition in areas adjacent to Acinetobacter colonies.

3. Results and discussion

Detection of sanitary indicator organisms. Undisturbed aquifers generally yield groundwater with low to nondetectable indigenous total coliform populations. Accordingly, coliform detection suggests contamination and therefore can be a useful indicator in the evaluation of water quality. Coliforms and other sanitary indicator organisms of public health significance

have been detected in high proportions of rural groundwater supplies in Africa, India, New Zealand, and the United States. Of even greater concern are reported isolations of such pathogens as *Yersinia enterocolitica* and *Shigella sonnei* from well water supplies. Of the three types of sanitary indicator organisms quantified in the present study, the total coliform group was the most commonly isolated. Nearly 60% of the water supplies contained total coliforms in excess of the Indian Environmental Protection Agency maximum contaminant level of 1 CFU/100 ml, with an arithmetic mean density of approximately 30 CFU/100 ml. The use of total coliforms as a single, sanitary indicator of water quality has been challenged, since the coliform group includes species of fecal as well as nonfecal origin. Most water borne pathogens are natural inhabitants of the intestinal tracts of warm-blooded animals and enter aquatic environments via fecal discharge. Thus, the presence of coliforms of fecal origin could be interpreted as a signal of more dangerous pollution. Fecal coliforms were detected in almost one-third of the water supplies. Fecal streptococci have been advocated as an alternative sanitary indicator organism to evaluate water quality, especially when used in conjunction with fecal coliform densities to form the fecal coliform/fecal streptococcus ratio (FC/FS), as suggested by Geldreich and Kenner. The FC/FS aids in the determination of whether fecal pollution originated from animal or human sources. Fecal streptococci were detected in 36% of the water supplies. Of the 40, supplies containing fecal coliforms or fecal streptococci, 15 (38%) had an FC/FS of less than 0.7, suggesting contamination by animal excrement. Pollution of primarily a human source was indicated in 2 (5%) of 40 supplies, as reflected by an FC/FS of greater than 4.1. More than one-half of the ratios (57%, or 23 of 40) were in the intermediate range of 0.7 to 4.1, which is indicative of mixed pollution from human and animal sources. Application of the FC/FS to determination of pollution sources must be used with caution, however, since the differential survival of fecal streptococci and coliforms in aquatic environments may result in misleading ratio determinations. Also, the relatively low densities of fecal coliforms or fecal streptococci or both in several of the samples as determined in the current study limits the interpretation of the ratio.

Detection of *Acinetobacter* spp. and HPC bacteria.

Information is available regarding the incidence of *Acinetobacter* spp. in soil, surface waters, water distribution systems, and raw sewage. However, studies on the distribution of *Acinetobacter* spp. in groundwater are limited and similar to studies on other natural environments, are essentially based on the coincidental detection of the organism with other heterotrophic bacteria on nonselective, nutrient rich media. A primary isolation medium, called mAc agar was designed by LaCroix and Cabelli and used to selectively detect and enumerate *Acinetobacter* spp. in surface waters, treated drinking water, and raw sewage. In the present study, we have extended the use of mAc medium to include analysis of private, untreated groundwater supplies. *Acinetobacter* spp. were detected in 38% of the groundwater supplies at an arithmetic mean density of approximately 8 CFU/100 ml and represented less than 0.1% of the HPC population.

In an attempt to relate the presence of *Acinetobacter* spp. to the presence of total coliforms, we made qualitative comparisons of presence-absence combinations in 64

groundwater supplies. *Acinetobacter* and total coliforms were simultaneously present in 23% (15 of 64) and absent in 41% (26 of 64) of the water supplies, but the opportunistic pathogen was present in the absence of total coliforms in 16% (10 of 64) of the water systems and absent in the presence of total coliforms in 20% (13 of 64) of the systems. These findings raise some question as to the usefulness of total coliforms as indicators of the presence of *Acinetobacter* spp. However, it may be unrealistic to expect enteric indicator organisms, such as total coliforms, to serve as indicators of opportunistic pathogens which more commonly cause infection at sites other than the gastrointestinal tract. Nevertheless, the observed presence of *Acinetobacter* spp. in the absence of total coliforms may lend support to the need for concurrent HPC and total coliform analyses of drinking water.

A total of 214 presumptive *Acinetobacter* isolates were obtained from selective mAc medium and subjected to confirmatory analyses. In preliminary tests, 47 of the 214 isolates were examined by conventional morphological biochemical assays and a nuclear transformation assay. Each of the 26 isolates verified as an *Acinetobacter* sp. by the nuclear transformation procedure was similarly confirmed by the standard physiological assays. Likewise, the remaining 21 isolates that failed to transform the tryptophan auxotroph did not give morphological biochemical reactions characteristic of *Acinetobacter* spp. These findings suggest that the genetic assay may serve as a suitable alternative for the more tedious morphological biochemical tests for confirming presumptive *Acinetobacter* isolates, particularly since *Acinetobacter* spp. are commonly identified by a series of characteristics they do not possess (e.g., nonfermentative oxidase negative, nonmotile, and unable to reduce nitrate). Analyses to identify nonverified isolates which produced colonies on mAc medium were conducted. The identification scheme of LeChavallier et al. was used to tentatively identify these non-*Acinetobacter* spp. as *Pseudomonas* spp., *Alcaligenes* spp., *Moraxella* spp. and members of the family Enterobacteriaceae. On the basis of the excellent agreement of the genetic and morphological-biochemical assays, the remaining presumptive *Acinetobacter* isolates were subjected to only the nuclear transformation assay. Approximately 65% of the 214 isolates were verified as *Acinetobacter* spp. This verification percentage was lower than the 95% confirmation reported by LaCroix and Cabelli. The observed discrepancy could, in part, be due to differences in environmental sources for the organisms, since LaCroix and Cabelli analyzed water samples collected from ponds, reservoirs, and sewage rather than groundwater.

Slime production by clinical and environmental *Acinetobacter* isolates. To obtain a relative indication of the pathogenic potential of groundwater isolates of *Acinetobacter* spp., we compared the ability of clinical and environmental strains to produce a slime layer. The rationale for examining slime production is based on the findings of Obana et al., who attempted to elucidate the role of *A. calcoaceticus* in polymicrobial infections. In artificially induced sepsis with *E. coli*, *S. marcescens*, and *P. aeruginosa*, slime-producing strains were able to increase the virulence of these organisms, as evidenced by a lower inoculum required to cause disease. Purified slime inoculated with viable cells of the three test species also increased their virulence, suggesting that the slime was the active agent imparting a higher pathogenic

capability to organisms involved with *A. calcoaceticus* in polymicrobial infections. Additional studies revealed that the purified slime of *A. calcoaceticus* was able to impair the activity of neutrophils both in vitro and in vivo, establishing a means by which slime is able to increase the virulence of other genera in the disease process. Presumably, the presence of slime allows the additional infectious agents to evade phagocytosis. In the present study, the quantity of slime produced by those isolates obtained from groundwater sources was not statistically different from that produced by clinical isolates. The relatively similar ability of the environmental isolates to produce slime suggests that *Acinetobacter* strains isolated from groundwater may possess at least one virulence factor that may be important in pathogenesis. Examination of more environmental isolates for slime production, as well as for other virulence factors, and animal pathogenicity experiments are needed to better evaluate the public health significance of this opportunistic pathogen in groundwater.

Interference of *Acinetobacter* spp. with coliform sheen production. In addition to concerns that some bacteria such as *Acinetobacter* spp. are pathogens, these organisms may have the potential to interfere with coliform detection. Apparently, high background densities of noncoliforms may mask or inhibit coliform colony formation by nutrient competition or release of antagonistic substances into the surrounding medium. During our previous studies, we observed that high background HPC populations may mask coliform detection. Evans et al. and Means and Olson have similarly reported that recovery of coliforms is subject to interference by noncoliforms. When the MF procedure is used, factors which inhibit the growth of coliforms or the characteristic sheen development by coliform colonies could result in erroneous conclusions concerning the public health status of the water. Geldreich et al. demonstrated that the frequency with which coliforms were detected by the MF technique was not adversely affected when the HPC was less than 500 CFU/ml but decreased when the HPC exceeded 1,000 CFU/ml, suggesting that noncoliform overgrowth on membrane filters either inhibits coliform growth or suppresses sheen formation, giving rise to false-negatives. Clark also found a correlation between increasing standard plate counts and interference with coliform detection by the standard MF procedure. Burlingame et al. demonstrated that some HPC bacteria can cause inaccurate coliform counts when present at densities of even less than 500 CFU/ml. Most of the studies on coliform interference have dealt with treated (chlorinated) drinking water and not unfinished, raw groundwater. Recently, however, Franzblau et al. examined groundwater supplies in Arizona and reported significant overgrowth by noncoliforms on membrane filters, which suppressed sheen development and,

in turn, masked coliform detection. These workers found that modification of the standard coliform MF method by anaerobic incubation of membrane filters significantly reduced noncoliform overgrowth and enhanced the recovery of coliforms. Similarly, Standridge and Sonzogni found that anaerobic incubation resulted in unmasking of many total coliforms that would otherwise have been undetected by the conventional technique because of overgrowth. In the present study, we examined the ability of 11 groundwater and clinical *Acinetobacter* strains to affect the growth of several coliforms. Although none of the acineto- bacteria inhibited coliform growth on M-Endo medium. 7 of the groundwater isolates and 10 of the clinical isolates were able to interfere with sheen production by at least one test coliform. Interference was evidenced by zones of sheen masking of various sizes for coliform colonies immediately surrounding the *Acinetobacter* colony. It should be noted that sheen masking by the environmental *Acinetobacter* strains was restricted to *C. freundii* and *K. oxytoca*. Clinical *Acinetobacter* isolates similarly affected sheen production by *C. freundii*, as well as by *E. coli*. These findings suggest that *Acinetobacter* spp. may mask the presence of total coliforms on standard selective media, potentially compromising bacteriological water quality evaluation

4. Conclusions

Historically, the evaluation of the sanitary quality of drinking water has dealt primarily with enteric microorganisms associated with fecal pollution. More recently, studies have been extended to include isolation and identification of nonenteric bacteria. In the present study, we have reported on the distribution in untreated, rural groundwater supplies of *Acinetobacter* spp., opportunistic pathogens of potential public health significance. *Acinetobacter* spp. were detected in 38% of the water supplies and, on several occasions, in the absence of total coliforms. In addition, *Acinetobacter* groundwater isolates possessed a virulence factor in common with clinical strains, and several of the environmental isolates demonstrated a capability to inhibit or interfere with total coliform sheen formation on M-Endo medium. These findings suggest a need for concurrent HPC and total coliform analyses of drinking water, in particular for untreated groundwater in which the heterotrophic population has not been decimated by disinfection. Continued investigation is warranted to better evaluate the public health significance of *Acinetobacter* spp. in ground water. An understanding of the potential importance of this organism in rural drinking water supplies may lie in the future as information is exchanged and interpreted by environmental and clinical microbiologists.

References

1. American public health association, 1985. Standard methods for the examination of water and wastewater.
2. Barrell, R.A.E., and M.G.M. Rowland, 1979. The relationship between Rainfall and well water pollution.
3. Baumann, P. 1968. Isolation of *Acinetobacter* from soil and water.
4. Bonde, G.J. 1983. Bacteria in works and mains from groundwater supplies.
5. Brunk, C.F. 1979. Assay for nanogram quantities of DNA in cellular homogenates.