Systematic and Applied Microbiology 38 (2015) 351–357

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Development and field evaluation of a method for detecting carbapenem-resistant bacteria in drinking water

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ARTICLE INFO

Article history:
Received 5 December 2014
Received in revised form 17 March 2015
Accepted 23 March 2015

Keywords:
Carbapenem resistance
Drinking water
New Delhi
Water testing
Antibiotic resistance
Method

ABSTRACT

In this study, a fluorogenic heterotrophic plate count test for drinking water was modified in order to detect the presence of carbapenem-resistant bacteria. Antimicrobial agents and concentrations were selected based on recoveries of known carbapenem-resistant and carbapenem-susceptible strains inoculated into simulated samples. The modified method was field-tested on 19 drinking water samples from the New Delhi, India distribution system. Samples exhibiting fluorescence indicated bacterial growth in the presence of the supplemented antimicrobial agents, and organisms from these samples were cultured. Twenty-one Gram-negative isolates were identified from nine of the 19 samples and the meropenem minimum inhibitory concentrations were determined. Ultimately, eight carbapenem-resistant organisms were isolated from five sampling sites within the New Delhi water distribution system.

Introduction

Infections associated with carbapenem-resistant bacteria, including community-acquired infections, are on the rise [18]. Since carbapenems are considered last resort drugs, few antibiotic options remain for carbapenem-resistant infections [9,13,15], and fatality rates as high as 50% have been reported in hospital-acquired cases [5,25]. Recent studies have isolated carbapenem-resistant bacteria from environmental samples, including treated drinking water [11,28,35]. The presence of carbapenem-resistant bacteria in drinking water suggests the potential for large outbreaks, gastrointestinal colonization, or both, resulting from massive dissemination of resistant bacterial contaminants via a water distribution system [18]. These disseminated organisms could hypothetically serve as a source of community-acquired carbapenem-resistant infections [14,19,26,28]. Therefore, there is a pressing need to develop methods to screen for the presence of such organisms, particularly in low-resource areas where carbapenem-resistant infections are prevalent.

Various methods exist for detecting antibiotic-resistant bacteria in water, including detecting resistance phenotypes using antibiotic selection on centrifuged or filtered samples [22,28,33] or direct testing for specific resistance genes using molecular methods [28]. Advantages of molecular testing include sensitivity, rapid results, resistance gene information that may be useful for epidemiological purposes, and the ability to perform multiplex assays that can identify multiple genes simultaneously. Disadvantages include the initial cost of equipment, the relative cost of molecular supplies, the need for operator technical expertise, and the potential presence of organic and inorganic PCR inhibitors in the environmental samples, which may become increasingly problematic when large volumes of water are concentrated and tested [12,20,31]. Concentration methods, such as sample centrifugation or membrane filtration, followed by bacterial enrichment on selective media, are commonly used to detect water contaminants in larger volumes of water, including antibiotic-resistant bacteria [28,30]. With

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; HPC, heterotrophic plate count; DI, deionized; PBS, phosphate buffered saline; MPN, most probable number; TSA, tryptic soy agar.

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http://dx.doi.org/10.1016/j.syapm.2015.03.010
0723-2020/

the exception of the initial costs of membrane filtration or centrifugation equipment, these methods can be cost-efficient and can generate live isolates for downstream testing (e.g. biochemical or molecular identification methods), but they can also be time-consuming and cumbersome when large numbers of samples are processed [4]. While these are each valid methods for detecting and isolating antibiotic-resistant bacteria from environmental sources, their disadvantages make them less practical for wide-scale environmental monitoring in lower income countries.

In the U.S., standard drinking water testing methods commonly utilize a chromogenic or fluorogenic substrate to test for the presence of coliforms and *Escherichia coli* in samples with volumes of at least 100 mL [2]. This method has previously shown promise in detecting antibiotic-resistant organisms when combined with selective antimicrobial agents [10].

However, assessing the potential impact of waterborne carbapenem-resistant bacteria on community-acquired infections requires methods that can capture a much wider array of organisms, as many of the bacteria harboring genes conferring carbapenem resistance are not *Enterobacteriaceae* and would not be detected by the total and fecal coliform tests commonly used in routine drinking water quality testing [4,24,28,32]. The presence of non-pathogenic heterotrophic bacteria may be captured by heterotrophic plate count testing; however, this testing is used to assess the adequacy of water treatment. The presence of heterotrophic bacteria in drinking water is not typically considered a human health concern [1,32], but the potential exists for such heterotrophs harboring resistance genes to spread via tap water [28]. Furthermore, if these antibiotic resistance genes are carried on mobile genetic elements, they may be transferrable to bacteria of the same or different species [3,17,21,23,28].

Consequently, there is a need for environmental testing methods that can detect the presence of carbapenem-resistant heterotrophic bacteria in drinking water. Therefore, the objective of this research was to develop, validate, and trial a low-cost, practical method for detecting a broad range of carbapenem-resistant bacterial species in drinking water samples.

Materials and methods

Method development

Four strains of *Klebsiella pneumoniae* and one strain of *Pseudomonas aeruginosa* were used as model carbapenem-resistant or carbapenem-susceptible bacteria for method development and validation. The included strains had different susceptibilities to meropenem. Two strains were meropenem-susceptible (*K. pneumoniae* ATCC 33495, MIC = 0.032 $\mu g\,mL^{-1}$; *P. aeruginosa* ATCC 27853, MIC = 0.25 $\mu g\,mL^{-1}$), and three strains were meropenem-resistant (two different KPC-producing *K. pneumoniae* strains with MICs of 16 $\mu g\,mL^{-1}$ and >32 $\mu g\,mL^{-1}$, and an NDM-1-producing *K. pneumoniae* (ATCC BAA-2146) with a meropenem MIC of >32 $\mu g\,mL^{-1}$). Minimum inhibitory concentrations were determined by the broth microdilution method using the Clinical and Laboratory Standards Institute (CLSI) *Method for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically* [6]. The results were interpreted using the 2014 CLSI *Performance Standards for Antimicrobial Susceptibility Testing* [7].

IDEXX heterotrophic plate count (HPC) reagent (IDEXX Laboratories, Westbrook, ME) was used in combination with specific antibiotics to selectively detect carbapenem-resistant *Klebsiella* and inhibit carbapenem-susceptible *Klebsiella* and *Pseudomonas*. Multiple concentrations of meropenem (U.S. Pharmacopial Convention, Rockville, MD) and vancomycin (Sigma–Aldrich, St. Louis, MO) were assessed in the development of the method. Vancomycin was added in order to increase the specificity of the test by suppressing the growth of Gram-positive organisms not inhibited by meropenem, including enterococci species and methicillin-resistant *Staphylococcus aureus* [34]. The IDEXX reagent uses multiple proprietary fluorogenic substrates to detect heterotrophic bacteria in drinking water samples. According to the manufacturer's instructions, positive samples are indicated by fluorescence at 365 nm after 44–72 h incubation at 34–38 °C; however, as meropenem stability in aqueous solutions decreases over time [8,16], counts after 44–48 h incubation were considered as the final result.

Single cell detection was assessed by comparing the recoveries of bacteria spiked into sterile deionized (DI) water samples containing only HPC reagent (no antibiotics) with samples containing reagent and various concentrations of meropenem. Overnight cultures (16–24 h) of the *Pseudomonas* and the four *Klebsiella* strains were inoculated into sterile phosphate buffered saline (PBS) in order to create a bacterial suspension with an optical density of 0.1 to 0.15, which was the equivalent of a 0.5 McFarland standard (approximately 1.5×10^8 cells). The suspension was serially diluted in sterile phosphate buffered saline down to 1.5×10^2 cells mL^{-1}, and 100 μL of each suspension (target ~15 cells) were spiked into 100 mL samples of sterile DI water containing the HPC reagent and 0.125, 1, or 4 $\mu g\,mL^{-1}$ concentrations of meropenem. HPC reagent was added first in order to establish a buffer prior to the addition of the spiked bacterial cells. Unspiked DI water controls containing reagent and the same antibiotic concentrations were also prepared. All organism/antibiotic combinations were tested in triplicate.

Bacteria were quantified using the IDEXX Quanti-Tray® most probable number (MPN) method (IDEXX Laboratories, Westbrook, ME). Samples were incubated at 35 °C and checked at 24, 48, and 72 h for fluorescence using long-wave UV (365 nm). The number of fluorescing wells were counted and the corresponding MPN was determined using the IDEXX Quanti-Tray® MPN table supplied by IDEXX. Mean MPN counts for each set of triplicate samples were calculated from the three individual sample MPNs after 48 h incubation. The percentage recovery for each sample set was calculated as the mean MPN count for each spiked sample set with antibiotic divided by the mean MPN count of the spiked samples without antibiotic, multiplied by 100. To verify the number of viable bacteria used to spike the samples, 100 μL of each diluted bacterial suspension were also plated on tryptic soy agar (TSA) in triplicate and the counts were averaged.

To determine if false positive fluorescence was occurring and to ensure that bacterial colonies could be isolated from the water samples, 20 μL of water from fluorescing wells of the DI water samples were extracted and plated onto CHROMagar™ KPC (DRG International, Springfield, NJ) and blood agar (Hardy Diagnostics, Salt Lake City, UT) for confirmation.

Method validation

For validation of the carbapenem-resistant bacteria screening method in a tap water matrix, unspiked tap water samples were tested in comparison with simulated water samples spiked using the same *Pseudomonas* and *Klebsiella* strains employed in the method development. Approximately 10–50 cells were spiked into the tap samples and tested with concentrations of 0.0625, 0.125, or 1 $\mu g\,mL^{-1}$ meropenem, and without meropenem. To increase the specificity of the test, 5, 6.25, 12.5, 25, or 75 $\mu g\,mL^{-1}$ vancomycin concentrations were combined with 1 $\mu g\,mL^{-1}$ of meropenem. These combinations were evaluated for their impact on the recovery of the NDM-1-producing strain and the suppression of non-target organisms. A concentration of 5 $\mu g\,mL^{-1}$ vancomycin was ultimately selected for use with three meropenem concentrations (0.0625, 0.125, or 1 $\mu g\,mL^{-1}$).