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Chlorhexidine resistance in a Gram-negative bacterium isolated from an aquatic source

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Abstract

Aeromonas hydrophila is a Gram-negative bacterium of considerable importance in both clinical, especially nosocomial infections, and zoonotic respects, both aquatic and terrestrial infections. In addition to the ability to thrive in a wide range of conditions, *A. hydrophila* is resistant to numerous antibiotics and antimicrobials. In conjunction with Kansas State University and the Kansas Water Office, water samples from various locations within Kansas were screened for organisms resistant to chlorhexidine. Chlorhexidine is the active agent in many surgical scrubs, prescription mouthwashes, and other bactericidal and bacteriostatic substances. *Aeromonas hydrophila* capable of growth in therapeutic levels of chlorhexidine was detected in one of the water samples. The isolate was determined not be harboring a plasmid, lending to a course of inquiry founded on the premise that the source of chlorhexidine resistance resides within the chromosome. Investigation into efflux pumps (EP) is underway with select resistance-nodulation-cell division (RND) pumps, especially the AheABC EP, being exculpated of responsibility for chlorhexidine resistance following testing with a select EP inhibitor. Inquiry into the major facilitator superfamily (MFS) EPs is underway with specific examination of the EmrB/QacA EPs, as chlorhexidine resistance in *Staphylococcus aureus* carrying the QacA gene has been observed.

Keywords

Chlorhexidine; *Aeromonas hydrophila*; EmrB; efflux pump; fluoroquinolone resistance

INTRODUCTION

Aeromonas hydrophila is a motile, non-spore forming, Gram-negative bacillus often associated with aquatic environments but with the capability of inhabiting a wide range of habitats of both a terrestrial and non-terrestrial nature.^[1,2] In addition to being a facultative anaerobe, it is relatively non-fastidious.^[3] Thermal tolerance of *A. hydrophila* ranges from 4°C to 45°C with viable growth in a pH range of 5 to 9.^[4] It also has the ability to produce biofilms as well as a number of toxins with varied natures.^[5,6] With the ability to survive chlorination lending it the ability to contaminate metropolitan water supplies as well as the

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exhibited resistance to select antimicrobial agents, *A. hydrophila* is worthy of investigation.^[7,8]

With its many ascribed qualities, *A. hydrophila* is capable of numerous maladies. Prior thought indicated pathological effects were primarily limited to fish and immunocompromised persons. As of late, this trend has been modified, with due cause, to include immunocompetent people as well. Pathogenesis is determined by route of infection. Introduction of *A. hydrophila* to a wound leads to massive and, in some cases, fatal infection often requiring amputation while ingestion leads to cholera like symptoms.^[9–10] Inhalation of this organism leads to hemorrhagic necrotizing pneumonia.^[11] On another note, the organism has also been implicated as a contributing agent to neoplastic disease.^[10] When the numerous pathological avenues are combined with the extensive virulence determinants such as biofilm production and multiple efflux pump systems, *A. hydrophila* is an organism worthy of consideration from both a scientific perspective and a healthcare aspect.^[6–12]

Surgical intervention of the body, from an intravenous catheter to surgery in the thoracic cavity, is an optimal time for an opportunistic pathogen to infect its host. Due to this, special attention to the prevention of colonization is given in medical and dental settings especially in the form of surgical scrub for surgeons and preparation cleanser for patients. Chlorhexidine is the active antimicrobial compound in many of the products used in previously described roles. In addition to this function, chlorhexidine is also used in an antimicrobial role in many other healthcare items to include implants and prescription mouthwashes.

Chlorhexidine (Fig. 1), a cationic biguanide, was introduced in 1954.^[13] It has shown great promise in a bactericidal role with little known resistance while maintaining low host tissue irritation.^[14] When considering Gram-positive and Gram-negative bacteria, greater levels of chlorhexidine are required for clinical dosage effectiveness when dealing with Gram-negative bacteria.^[13] Chlorhexidine exposure results in cellular membrane rupture followed by death of the bacteria with coagulation of intracellular components and congealing of the cytoplasm.^[14]

Bacteria have evolved many methods of resistance to antimicrobials/antibiotics. One mechanism that has come to the forefront of research, as of late, are efflux pumps. Individual structures and arrangements of these pumps vary to a great degree. The overall function, extrusion of toxic substances from the organism, does not. There are, currently, five recognized families of efflux pumps.^[15] Of special note, in the healthcare/antibiotic campaign, are the ATP-binding cassette superfamily (ABC), the major facilitator superfamily (MFS), and the resistance-nodulation-cell division superfamily (RND).^[15] The ABC pumps are driven by ATP hydrolysis while MFS and RND pumps are proton antiporters.^[15] In addition to being the largest family of efflux pumps, it should be noted that MFS efflux pumps often operate, in Gram-negative bacteria, as part of a tripartite systems for extracellular efflux of substances due to cell wall structure inherent to Gram negative bacteria.^[15]

The objectives of this investigation were four-fold. The first of these aims was to screen environmental water samples for organisms resistant to chlorhexidine at therapeutic concentrations. The second purpose was to investigate additional resistance properties of any isolates. The third point was to determine if any isolates were harboring plasmids with resistance markers. The fourth and final intent of this inquiry was to determine the origin of the chlorhexidine resistance.

MATERIALS AND METHODS

Sampling and culturing protocols

Water samples were collected weekly from thirty-three sites in west central Kansas (Fig. 2). Samples were screened weekly for organisms capable of growth in a therapeutic concentration of chlorhexidine, 0.02%. This was accomplished through combining environmental water samples with 2X tryptic soy broth with a 0.04% chlorhexidine digluconate concentration in a one to one ratio. Samples were incubated at 37°C overnight. Growth was determined through observation of turbidity by visual examination.

Isolate identification

The sample that gave a positive indication of growth in 0.02% chlorhexidine was subcultured by three passages on 0.02% chlorhexidine tryptic soy agar. Upon overnight incubation at 37°C, a Petri plate containing the pure culture was sealed with parafilm and sent to MIDI Labs (Newark, Delaware). Identification of the isolate took place through 16S DNA alignment. A report of identification to the subspecies level was received electronically.

Antibiotic/antimicrobial susceptibility assessment

Antibiotics utilized included ciprofloxacin, gentamicin, tetracycline, vancomycin, ampicillin, and azithromycin. Initial screening of the chlorhexidine resistant isolate for antibiotic susceptibility was determined through Kirby-Bauer Assay on Mueller-Hinton agar with the bacterial inoculum conforming to a 0.5 McFarland Standard. Specific antibiotic minimum inhibitory concentration (MIC) was established through the use of Etest strips (BioMerieux, Durham, North Carolina) on Mueller-Hinton agar with bacterial inoculum conforming to a 0.5 McFarland Standard. In all cases, media were inoculated through the employment of sterile swabs.

The chlorhexidine resistant isolate was screened for resistance to the antimicrobial substance triclosan. A minimum inhibitory concentration assay utilizing dilution of a tryptic soy broth/triclosan mixture was accomplished. The highest concentration of triclosan used was 0.15%. Corresponding triclosan concentrations were diluted in a 1:10 ratio.

Plasmid extraction protocols

Techniques attempted for extraction of possible plasmids included commercial and high volume plasmid extraction procedures. Specifically, the Wizard Plus SV Minpreps system (Promega, Madison, Wisconsin) was used as well as the alkaline lysis extraction

procedure.^[16] Resultant extracts were subjected to electrophoresis in agarose gels with densities ranging from 0.5% to 1%. Periods of electrophoresis ranged from 90 minutes to 240 minutes. Staining of agarose gels was accomplished via staining in an ethidium bromide. Images of the stained agarose gels were captured under ultraviolet illumination, optimized for clarity, with a Kodak GL-100 gel system.

Resistance-nodulation-cell division efflux pump inhibition analysis

RND efflux pump inhibition testing was accomplished via dilution plate technique utilized by Rajendran et al. in investigation of efflux pumps and *Burkholderia*.^[17] In this inquiry, a mixture of chlorhexidine and efflux pump inhibitor, phenylalanine arginyl β -naphthylamide (PA β N), in tryptic soy broth was used. Four 24 well plates were set in a fashion to form a 96 well plate arrangement (Fig. 3). The left column of wells did not contain chlorhexidine. From left to right, on the y-axis, concentrations of chlorhexidine started at 0.02% and were diluted in a 1:2 ratio. The top row did not contain any efflux pump inhibitor. The second row contained PA β N at a concentration of 512 mg/L. Concentrations of PA β N declined in a concentration decreasing each row, on the x-axis, in a manner of each subsequent row being the result of the previous concentration divided by two with the bottom row of wells containing 8 mg/L.

RESULTS

Water sample screening

Water sample screening revealed one isolate positive for growth in the presence of 0.02% chlorhexidine. Light microscopy and Gram staining revealed Gram-negative bacilli grouped in singles and pairs (Data not shown).

Isolate identification

Sequencing and alignment of the first 500 base pairs of the 16S DNA identified the isolate as *Aeromonas hydrophila hydrophila* with 100% confidence.

Antibiotic/antimicrobial susceptibility

The isolate demonstrated a minimum inhibitory concentration (MIC) for ciprofloxacin in excess of 32 $\mu\text{g mL}^{-1}$. The MIC for gentamicin was 0.25 $\mu\text{g mL}^{-1}$ and tetracycline was 48 $\mu\text{g mL}^{-1}$. The MICs for both vancomycin and ampicillin were in excess of 256 $\mu\text{g mL}^{-1}$. The MIC for azithromycin was 16 $\mu\text{g mL}^{-1}$. The MIC for triclosan was in excess of 0.15% (Table 1).

Plasmid extraction

None of the plasmid extraction procedures indicated the presence of any plasmids within the isolate (Data not shown).

Resistance-nodulation-cell division efflux pump assay

The resistance-nodulation-cell division efflux pump inhibition assay did not reveal any change in the MIC of the isolate to chlorhexidine.

DISCUSSION

The presence of *Aeromonas hydrophila* with chlorhexidine resistance in a natural water source in Kansas is of concern, with potential significance to science and healthcare. Chlorhexidine resistance is relatively unknown in Gram-negative bacteria.

With the elimination of the possibility of the isolate harboring a plasmid, it is likely the chlorhexidine resistance is encoded chromosomally. Chlorhexidine resistance has been noted, to a limited degree, in Gram-positive bacteria by means of the MFS QacA efflux pump.^[18] This efflux pump is highly similar to the MFS EmrB efflux pump, in select Gram-negative bacteria, in both structure, both having 14 membrane spanning alpha-helices, and in mass, 55 kDa and 56.2 kDa respectively.^[18,19] In fact, it has been noted that the translated amino acid sequence of EmrB is homologous to QacA.^[19] The type strain of *Aeromonas hydrophila*, ATCC 7966, is known to have the EmrB efflux.^[20] While the EmrB efflux pump is part of a tripartite complex due to the structural difference of the cell wall between Gram negative and Gram positive bacteria (Fig. 4), current literature indicates a measure of congruity between it and the QacA efflux pump.^[18,19]

The RND efflux pump inhibition assay revealed that the AheABC and MexAB RND efflux pumps, mechanisms responsible for a considerable segment of multidrug resistance among *A. hydrophila*, are not the culprits behind the chlorhexidine resistance.^[16] We hypothesize a potential mechanism of chlorhexidine resistance in our isolate is a MFS efflux pump EmrB, or a closely related structure. Future studies will involve further delineation of the mechanism. While the resistance does not appear to be plasmid-mediated, it could be transferable to other bacteria, since *Aeromonas hydrophila* may act as a reservoir of antibiotic resistance genetic elements.^[15]

While the scientific aspects of this investigation are of scientific interest, the results are also a potential concern to healthcare. Chlorhexidine, when complexed with any number of inert substances, is a primary method of infection control in many areas especially in the surgical fields from pre-op cleansing of the patient to the pre-op scrub of the surgical staff. This chief means of infection prevention in a medical/dental setting could be rendered ineffective if chlorhexidine resistance becomes widely disseminated.

The discovery that our isolate also shows marked resistance to ciprofloxacin is another potential cause for concern. Current treatment protocols for *A. hydrophila* infection, as indicated within the Johns Hopkins Antibiotic Guide, call for the use of quinolones, especially ciprofloxacin. This treatment, in the past, was likely appropriate as publications as recent as 2010 indicate near universal susceptibility to quinolones.^[21] With the discovery of the sample being completely resistant to ciprofloxacin, the prescribed treatment would be ineffective. With an established mortality rate of 50% for patients afflicted with *Aeromonas*

pneumonia, this is significant.^[21] Prescribed ancillary treatment would indicate gentamicin as an appropriate choice.^[22] While antibiotic susceptibility of the isolate indicated this would likely be effective, the high toxicity of gentamicin when combined with the presence of mobile gentamicin resistance elements in the environment as well as a significant percentage of environmental *A. hydrophila* isolates being resistant, one would be led to the conclusion that current treatment protocols may be inadequate.^[22–24]

With the observation of a previously unknown chlorhexidine resistance in Gram-negative bacteria and the detection of a relatively unusual resistance to ciprofloxacin in the organism, both the scientific world, especially within the research of resistance armamentaria of bacteria, and the healthcare sphere have a significant impetus to continue investigation into these findings as the impact within their respective realms may not only affect knowledge within the fields but have impacts on mortality and morbidity of patients. Future investigation may include confirmation of the EmrB gene within the isolate as well as the location or creation of a suitable efflux pump inhibitor to test the EmrB efflux pump system through means previously ascribed in this work in reference to RND efflux pump inhibition testing. The penultimate confirmation of the EmrB/chlorhexidine resistance link could include the creation of a mutant strain of *A. hydrophila* lacking the EmrB gene with a corresponding loss or reduction of chlorhexidine resistance. *Aeromonas hydrophila* is indeed an organism worthy of study for the benefit of science and healthcare and merits further investigation.

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Figure 1.
Structural formula of chlorhexidine- $C_{22}H_{30}Cl_2N_{10}$. Courtesy NCBI

Figure 2.
Water sampling locations-west central Kansas, USA. Dots indicate sample locations. FHSU 6 denotes sampling site where the chlorhexidine resistant isolate was collected. Due to scale, not all sample sites are shown.

Figure 3. RND efflux pump inhibition assay. The left column of wells did not contain chlorhexidine. From left to right, on the y-axis, concentrations of chlorhexidine started at 0.02% and were diluted two-fold. The top row did not contain any efflux pump inhibitor. The second row contained PAβN at a concentration of 512 mg/L. Concentrations of PAβN declined in a concentration decreasing each row.

Figure 4. Diagrammatic representation of hypothesized efflux pump positioning and function in their respective classes of bacteria. OM denotes outer membrane. CM denotes cell membrane.

Table 1

Chlorhexidine resistant isolate minimum inhibitory concentrations (MIC) as determined by Etest strips.

Antibiotic	MIC (µg/mL)
Ampicillin	>256
Azithromycin	16
Ciprofloxacin	>32
Gentamicin	0.25
Tetracycline	48
Triclosan	>0.15*
Vancomycin	>256

* The triclosan MIC is expressed as % (w/v) and was determined by broth dilution.