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Presence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in sewage treatment plant

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HIGHLIGHTS

- Presence of Methicillin Resistant *Staphylococcus aureus* (MRSA) was explored in a rural sewage treatment plant.
- MRSA was present in raw and treated sewage.
- The isolated bacteria carried *mecA* gene responsible for methicillin resistance.
- Free DNA of *mecA* gene is released from the sewage treatment plant.

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ABSTRACT

The presence of antibiotic resistant bacteria and antibiotic resistance genes in rural sewage treatment plants are not well reported in the literature. The aim of the present study was to study the frequency occurrence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in a rural sewage treatment plant. This study was conducted using raw sewage as well as treated sewage from a small town sewage treatment plant in rural southeast Louisiana of USA. Results showed the presence of MRSA consistently in both raw and treated sewage. The presence of *mecA* gene responsible for methicillin resistance was confirmed in the raw and treated sewage water samples.

1. Introduction

In the long list of monitored and researched health risks, antibiotic resistance is making its way back to forefront of concern. Antibiotics were once touted as miracle drugs that could cure formerly fatal infections, but as the use of antibiotics has increased to overwhelming proportions, so has the exposure and contact of microbes to these drugs. According to the Center for Disease Control, each year at least 2 million people become infected with bacteria that are resistant to antibiotics in the United States alone, and at least 23,000 people die as a direct result of those infections (CDC, 2015). Currently, antibiotics are the most widely prescribed and successful pharmaceuticals used for human medicine (Bouki et al., 2013). Although there are now more than 22 classes of antibiotics (Coats et al., 2011), bacteria are able to evade their bactericidal properties by evolving means of enzymatic degradation of antibacterial drugs, alteration of bacterial proteins that are antimicrobial targets, and changes in membrane permeability to antibiotics (Dever and Dermody, 1991). While various genes conferring antibiotic resistance may be intrinsic to certain species of bacteria, others gain this ability from bacteria to bacteria through plasmid-mediated conjugation, from free DNA through genetic transformation, and from viruses through viral transduction. In addition, these effects are exacerbated by humans' improper prescription, administration, and disposal of antibiotics. Acts such as taking too many prescription antibiotics, exceeding pharmaceutical dosage recommendations, excessively using the drugs prophylactically, taking antibiotics for viral infections, not complying with prescriptions, and self-prescribing antibiotics all contribute to resistance by over-exposing cultures to these bactericidal or bacteriostatic chemicals. Overexposure allows for those specimens that have become resistant to thrive and proliferate within an environment that is newly freed of susceptible isolates. Many staple antibiotics in physicians' repertoires are no longer functional against bacteria, such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) and

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Carbapenem Resistant Enterobacteriaceae (CRE), which forces them to resort to more extreme, broad-spectrum options.

Antibiotic resistance in hospital and clinical setting is well reported, however, the antibiotic resistance in the environment especially in rural USA is not well reported. Sewage treatment typically involves three major treatments, including a primary treatment to remove suspended solids, a secondary treatment to remove organic matter, and a tertiary treatment to disinfect pathogens (Cowan and Talaro, 2006). In the primary treatment, larger floating materials are skimmed off of the top of the raw sewage. Sedimentation tanks are used to remove suspended particulate matter. In the secondary treatment, organic matter is digested by a community of microbes through a process known as activated sludge. Biodegradation typically produces solid wastes, known as sludge, which collect at the bottom of the tank and break down slowly. In order to break down the sludge more quickly, the sludge is activated through the injection of air, mechanically stirred, and recirculated. In the tertiary treatment, the wastewater is disinfected and discharged. The focus of this study was to find the prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in a small-scale rural sewage treatment plant. The City of Thibodaux in the state of Louisiana, USA is a rural community with a population of 15,000. The sewage treatment in Thibodaux employs a traditional three-stage sewage treatment system as mentioned above along with an anaerobic digester to treat the sludge. The treated wastewater is pumped to a nearby wetland to be further purified by the environment (Goldstein et al., 2012). Prior to discharge to the wetland, the wastewater passes through an ultraviolet disinfection system to reduce microbial load in the water samples.

Methicillin, first introduced in 1959, is a penicillin-like antibiotic that is resistant to the action of penicillinases. Methicillin was used as an effective treatment for infections caused by *S. aureus*; however, within a year of the introduction of methicillin, Methicillin-Resistant *S. aureus* (MRSA) were reported (Gordon and Lowy, 2008). After careful analysis, methicillin resistance was shown to be conferred by the *mecA* gene, a gene, which codes for a mutated penicillin-binding protein called penicillin-binding protein 2a (PBP2a) (Morell and Balkin, 2010). This protein, unlike the original penicillin binding proteins, has a low affinity for beta-lactams like methicillin.

From 1960 until the early 1990s, Methicillin-Resistant *Staphylococcus aureus* were generally associated with hospital settings; however, in the early 1990s, community-associated strains of MRSA (CA-MRSA) emerged (Gordon and Lowy, 2008). CA-MRSA are resistant to the action of methicillin and have a gene that codes for a toxin that hospital-acquired MRSA cannot produce. Both hospital-acquired Methicillin-Resistant *Staphylococcus aureus* (HS-MRSA) and community-associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) pose significant health risks to human populations. According to Klein et al. (2007), approximately 5500 people die every year from MRSA-related infections, and MRSA is the leading cause of lower respiratory tract infections and surgical site infections. One reason that MRSA have become a major source of dangerous infections is that all *S. aureus* are hardy organisms that are capable of living in a wide variety of environments. *S. aureus* colonization is not limited to humans; *S. aureus* has been isolated from cats, dogs, pigs, and cows. The number of outbreaks of CA-MRSA has steadily increased over recent years among individuals who share close contact with others (Goldstein et al., 2012). Although HA-MRSA strains have traditionally been associated with hospital settings and CA-MRSA strains are usually found in community settings, the two populations of MRSA are beginning to intermingle (Gordon and Lowy, 2008). For these reasons, reducing the amount of contact individuals have with any MRSA strains is of utmost importance if the MRSA is to be controlled.

Sewage treatment plant is an ideal habitat for the development of antibiotic resistant bacteria as large numbers of bacteria come in close contact along with many types of antibiotics discharged in the wastewater (Everage et al., 2014). The purpose of this study was to test samples from the Thibodaux sewage treatment facility for the presence of Methicillin-Resistant *Staphylococcus aureus* (MRSA). The specific objectives of this study were to survey MRSA populations in raw and treated sewage and to confirm the presence of MRSA gene (*mecA*) using molecular techniques. The novelty of this study is the survey on the antibiotic resistant bacteria and the antibiotic resistance genes in sewage treatment plant in a rural area in USA, which was reported very rarely.

2. Methods

2.1. Sample collection

Monthly samples were collected from the Thibodaux sewage treatment plant, Thibodaux, Louisiana, USA for a period of 12 months from January 2015 to December 2015. Samples were collected from raw sewage that come into the plant and the treated sewage that is discharged to the wetland. The collected samples were brought to the lab in a cooler and processed immediately.

2.2. Quantification of Staphylococcus aureus in the sample

The *S. aureus* in the raw sewage and treated sewage were quantified every month using the Manitol salt agar (MSA) media, which is selective and differential for the presence of *S. aureus* (Leboffe and Pierce, 2010) and it produces yellow color colonies in MSA. The samples were serially diluted and the dilutions from 10^1 to 10^8 were plated in Petri dishes using pour plate technique with MSA to obtain countable colony forming units (CFU). The plates were incubated at 37 °C for 24 h and the yellow colonies were counted and reported as CFU/ml of sample.

2.3. Isolation of Staphylococcus aureus

To isolate *Staphylococcus aureus*, sterile cotton swabs were used to transfer bacteria from the water sample collection vessels to tryptic soy broth (TSB) tubes (MP, Solon, OH) according to the procedure described by Leboffe and Pierce (2010). The TSB tubes were incubated at 37 °C for 24 h. From the TSB cultures, a small amount of sample was transferred to mannitol salt agar (MSA) plates (Remel, Lenexa, KA) using sterile cotton swabs. An inoculation loop was used to isolate pure culture using the quadrant streak method. The MSA plates were incubated at 37 °C for 24 h. After twenty-four hours, one yellow colony (yellow colony in MSA medium indicates the presence of *S. aureus*) from each of the MSA plates was transferred to TSB. The TSB tubes were incubated at 37 °C for 24 h. These TSB tubes were treated as pure cultures for the duration of the experiment. For long-term storage, the isolated bacteria from the pure TSB cultures were transferred to TSA slants, and the TSA slants were incubated at 37 °C for 24 h and after incubation were stored at 4 °C.

2.4. Kirby-Baur disk diffusion assay

The isolated pure culture was tested every month for antibiotic resistance using vancomycin, which is in methicillin family of antibiotics. A small amount of the pure culture was pipetted into a sterile test tube and the turbidity of culture was adjusted using