Presence of Antibiotic Resistant Staphylococcus aureus in Sewage Treatment Plant

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ABSTRACT
Antibiotic resistance has become very common in the world. After passing through the human or animal body the antibiotics are entered into the sewage treatment plant, where water is processed and cleaned then returned into the environment. During the sewage treatment process, antibiotics come into contact with bacteria entering the treatment process, as well as bacteria used in the treatment process. The bacteria that are exposed to these antibiotics can become resistant during the treatment process and then expose the antibiotic resistance genes (ARGs) to the environment upon release of treated water from the treatment plant. Because of the contact between bacteria and antibiotics during the treatment process, sewage treatment plants are considered prime habitat to create antibiotic resistant bacteria. There are very limited studies on this subject from a small town sewage treatment plant. Therefore, this study was conducted using raw sewage as well as treated sewage from Thibodaux sewage treatment plant, which serves 15,000 people in rural southeast Louisiana of USA. Samples were collected monthly from Thibodaux sewage treatment plant and antibiotic resistance was monitored using Kirby-Bauer assay. Special attention was given to Methicillin Resistant Staphylococcus aureus (MRSA) in raw and treated sewage samples for the five month of the study period. 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 isolates of pure culture of S. aureus as well as in the sewage samples.

Keywords: Sewage treatment; antibiotic resistant; mecA gene, free DNA; Staphylococcus aureus; genetic transformation

1. INTRODUCTION

Staphylococcus aureus is a Gram positive human pathogen that is responsible for dangerous infections in individuals around the world. S. aureus belongs to a group of bacteria that normally live on the surface of the skin of humans. According to Gordon and Lowy (2008), approximately 30% of individuals are intermittently colonized with S. aureus, and approximately 20% of individuals are persistently colonized. S. aureus is resistant to penicillin, an antibiotic that disrupts a protein (penicillin-binding protein) that is essential to the synthesis of bacterial cell walls. S. aureus produces a protein called penicillinase that binds to penicillin and disrupts the antibiotic’s chemical structure (Morell and Balkin, 2010).

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 5,500 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.

The focus of this study is to find the prevalence of *S. aureus* in a small scale rural sewage treatment plant. 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 (Cowan and Talaro, 2006). Sedimentation tanks are used to remove suspended particulate matter (Robens, 1996). 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 (Cowan and Talaro, 2006). In order to break down the sludge more quickly, the sludge is activated through the injection of air, mechanically stirred, and recirculated (Cowan and Talaro, 2006). In the tertiary treatment, the wastewater is disinfected and discharged (Cowan and Talaro, 2006).

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 eliminate any microbes present in the water samples.

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. Elimination of the bulk of bacterial load occurs
in the disinfection step of the sewage treatment process; however, many problems exist with current methods of disinfection. Many sewage treatment facilities employ a chlorination system, but chlorination systems are expensive (Robens, 1996). As a result some sewage treatment facilities have switched to an ultraviolet disinfection system. Ultraviolet light destroys the DNA of microbes, thus, preventing microbes from reproducing. However, ultraviolet light may induce the production of heat shock proteins, proteins which repair DNA damage and guards against apoptotic processes (Behzadi and Behzadi, 2012). Inefficient treatment processes, including incomplete disinfection, are a major method of microbial introduction to the environment (Hendricks and Pool, 2012).

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.

2. METHODS

2.1 Sample collection

Monthly samples were collected from the Thibodaux sewage treatment plant, Thibodaux, Louisiana, USA for a period of five months from October 2013 to February 2014. 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 $10^1$ - $10^5$ 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 hours 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 hours. 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 (Leboffe and Pierce, 2010). The MSA plates were incubated at 37°C for 24 hours. 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 hours. These TSB tubes were treated as pure cultures for the duration of the experiment. For long-term storage of isolated bacteria, bacteria from the pure TSB cultures were transferred to TSA slants, and the TSA slants were incubated at 37°C for 24 hours 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 resistant using Methicillin.
A small amount of the pure culture was pipetted into a sterile test tube and the turbidity of culture was adjusted using sterile saline to match the turbidity of the McFarland standard (Leboffe and Pierce, 2010). Using a sterile cotton swab, a bacterial lawn was created on a MSA plate and Methicillin antibiotic disc was dispensed onto the surface of the MSA plate. The plate was incubated at 37°C for 24 hours. After the incubation time, the zone size was measured in millimeter using a manual caliper. The zone size was compared to interpretive standards to examine the antibiotic susceptibility of the isolated bacteria.

### 2.5 Molecular analysis of mecA gene

The presence of mecA gene in the raw and treated sewage as well as from the isolated S. aureus was analyzed using the mecA primer, 5′ATGCGCTATAGATTGAAAGGAT-3′ as demonstrated by Suzuki et al. (1992). The primer was obtained from Sigma Aldrich Co., (St. Louis, USA). Raw sewage and post UV treated sewage samples were centrifuged at 13,000 rpm for 15 minutes. The pellet was transferred to a 1.5 ml microfuge tube and a FAST ID DNA extraction kit was used to extract the DNA according to the instructions provided by the manufacturer. Similar procedure was followed for the pure cultures of S. aureus. After the DNA was extracted, polymerase chain reaction (PCR) technique was used to amplify the DNA with 5 µL DNA extract, 32.5 µL DNA free water, 1 µL of forward primer, 1 µL reverse primer, 5 µL deoxynucleoside triphosphates, 5 µL buffer, and 0.5 µL taq polymerase. The tubes were then placed in the PCR machine (Life Technologies, Applied Biosystems, Grand Island, NY; model number 2720) and allowed the cycle to completion. The PCR cycle was similar to the one described by Everage et al. (2014). A 2% agarose gel with ethidium bromide was prepared and used to visualize the PCR samples. 10 µL PCR sample was mixed with 2 µL 6x loading dye and injected into each well. The gel was run at 100 V for an hour. The gel was visualized using FluorChem FC2 imaging system.

### 2.6 Statistical analysis

All data were subjected to an analysis of variance (ANOVA) test (p ≤ 0.05) followed by a tukey “post hoc” analysis.

### 3. RESULTS AND DISCUSSION

#### 3.1 Presence of S. aureus in raw and treated sewage

S. aureus was consistently present in both raw and treated samples in all the sampling periods. Fig. 1 shows the bacterial load during the various sampling periods. As expected the population of S. aureus was high in raw sewage compared to treated sewage and it was statistically significant (p< 0.05). The Thibodaux sewage treatment uses UV light as a disinfecting agent before the treated sewage was discharged into the nearby wetlands. Even though the UV light removed most of S. aureus, the sample contained on an average around 1000 CFU/ml. S. aureus in the sample was positively identified by using the selective and differential medium of MSA. MSA is selective and differential for S. aureus as it contains 7.5% salt which will eliminate most of the bacteria and the substrate manitol in the medium is only metabolized by S. aureus, which will produce fatty acid from manitol. The fatty acid turns the medium to acidic and the pH indicator present in the medium will turn yellow color under acidic condition (Duguid, 1989). The presence of yellow color colony positively identifies the presence of S. aureus in the sample.
**Figure 1**  *S. aureus* population in raw and treated sewage for each month of sampling during 2013-2014 (Data represent average of two samples per sampling event. S.D was less than 5%)
3.2 Methicillin resistance

The isolated *S. aureus* from the sewage sample was tested for the antibiotic resistance, specifically to the antibiotic, Methicillin to see whether MRSA is prevalent in sewage. The result indicated MRSA is commonly present in both raw and treated sewage (Fig. 2). The antibiotic resistance was significantly higher with a p value of < 0.05 in treated sewage than sewage. This implies that the bacteria are picking up antibiotic resistant gene during the treatment time when the sewage was held for a hydraulic retention time (HRT) of five days. During this HRT, the activated sludge is an ideal place with bacteria habituating in close
quarters to go through bacterial gene transfer in one or several methods such as genetic transformation, conjugation, and transduction (Everage et al., 2014). When different species of bacteria grow in close proximity to each other, the growing bacteria produce compounds not normally produced when they grow alone (Traxler et al., 2013). Some bacteria can produce extracellular enzymes to deactivate the antibiotic, allowing sensitive bacteria the chance to grow in the environment (Salyers and Whitt, 2001). Sensitive bacteria can also coexist with resistant bacteria in the presence of antibiotics by covering the sensitive bacteria with a layer of resistant bacteria (Narisawa et al., 2008). In this study, the possible mechanism for antibiotic resistance is the genetic transformation as mecA gene was present in the treated sewage as a free DNA, which might have played a key role in conferring antibiotic resistance to bacteria that are sensitive to antibiotics as described by Everage et al. (2014).

### 3.3 Presence of mecA gene

To positively confirm the presence of mecA gene, which is the gene that confers the resistance to Methicillin, molecular analysis was done in raw and treated sewage samples. The mecA gene was consistently observed in both raw as well as treated sewage. Fig. 3 shows the result of mecA assay for the month of February 2014 as an example. Lane 1 in the gel is the raw sewage sample, lane 2 represents treated sewage, lane 3 is the negative control with deionized water, and lane 4 is the positive control with the primer of mecA gene. From Fig. 3, we can positively identify the presence of mecA gene in both raw and treated sewage sample. This was consistent for all sampling periods. Moreover, the isolated colonies also showed the presence of mecA gene as indicated in Fig. 4. This figure shows various S. aureus isolates for five months and all the isolates showed positive band for the mecA gene. This result confirmed S. aureus isolated from the sewage sample as MRSA. Suzuki et al. (1992) showed the presence of mecA gene in S. aureus and also in S. epidermidis. Genetic material that confers methicillin resistance may be passed from one organism to another through a process known as transformation in which free DNA from one organism is taken up by another organism and as a result develop antibiotic resistance. UV light kills most bacteria and at the same time may promote the release of free DNA into the wastewater as shown in this study. Thus through transformation process, bacteria may acquire antibiotic resistance trait.

The purpose of this study was to investigate the presence of ARGs in the form of mecA gene and MRSA in raw and treated sewage sample and the results clearly showed the presence of not only MRSA, but the presence of free mecA gene floating in the raw and treated sewage. This antibiotic resistance could potentially spread to the native bacteria present in the wetland where the treated sewage is discharged. This study demonstrated that the free DNA from the treated sewage, which contained the mecA gene is the main source for antibiotic resistance in the natural environment. Other studies have also shown that wastewater treatment plants are a common source of resistance genes (Everage et al., 2014; LaPara et al., 2011) to the natural environment.

Antibiotics are among the most commonly used and successful group of pharmaceuticals used for human medicine (Bouki et al., 2013). Therefore, rapid spread in resistance to these antibiotics has caused medial concerns to both public and health professionals. 1 in 5 trips to the emergency room (ER) are due to side effects of antibiotics. At least 2 million people become infected with resistant bacteria each year in the US and at least 23,000 die directly as a result, resulting in a cost of over $30 billion (CDC, 2014). Resistance is a result of
both the appropriate use of antibiotics, such as normal exposure due to usage, and inappropriate use, such as not finishing a prescription or over-use of the drugs. Other reasons include the selective pressure of antibiotic use, as well as technical changes that enhance the transmission of resistant organisms. There are many modes of resistance, such as decreased permeability, altered target site, or enzyme inactivation. MRSA is a commonly discussed resistant bacteria in public health. More people die annually in the US from MRSA than AIDS and homicides combined (Spellberg et al., 2011). The goal is to slow down the rise in antibiotic resistance genes (ARGs) by implementing better hygiene, preventing infections, controlling the nosocomial transmission of organisms, treating the source of the causative agent, and changing and developing new treatment methods (Dzidic et al., 2003).

Recent studies show that incomplete metabolism in humans and improper disposal of antibiotics to sewage treatment plants has been a main source of antibiotic release into the environment (Rizzo et al., 2013). This gives bacteria enough time and sufficient contact to shield themselves by altering their genes and cellular mechanisms, favoring their growth and reproduction (Galvin et al., 2010). These genes can go on to infect the wildlife in the estuaries when the treatment plants discharge their treated wastewater. Since 2007, over 3 million hunting and fishing licenses have been sold in Louisiana (Louisiana Wildlife and Fisheries, 2013). This has the potential to spread to humans that come into contact and consume the wildlife here in the wetland, where the sewage is discharged. More studies are needed to broaden other antibiotic resistance such as tetracycline, erythromycin, streptomycin, and sulfanilamide, which are some of the common antibiotics prescribed in Thibodaux area clinics.

CONCLUSIONS

This study clearly demonstrated the prevalence of \textit{S. aureus}, MRSA, and free DNA of \textit{mecA} gene in raw and treated sewage samples of Thibodaux treatment plant. Bacterial load was higher in raw sewage compared to treated sewage. However, the antibiotic resistance was higher in treated sewage than raw sewage. This is due to the ideal condition in the sewage treatment plant for bacteria to develop antibiotic resistance. The \textit{mecA} gene was present both in raw and treated sewage in all sampling periods. MRSA was identified in all the pure culture isolates isolated from sewage samples. Thibodaux sewage treatment plant is operating according to its designed purpose, which is to remove organic carbon. Sewage treatment plants are not designed to remove antibiotic resistance genes. This is an emerging problem and it should be addressed by public health officials.

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REFERENCES


