Antibiotic resistance of *Escherichia coli* isolated from a stream near two wastewater treatment facilities in Edmond, Oklahoma.

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***Escherichia coli* were collected from a stream in Edmond Oklahoma that receives effluent from two wastewater treatment facilities. *Esherichia coli* isolates collected at effluent and downstream of effluent had significantly smaller mean zones of inhibition compared to isolates collected upstream of effluent to several antibiotics tested. The percentages of isolates resistant to at least one or more antibiotics were progressively higher at effluent and downstream of effluent compared to isolates collected upstream of effluent. Thus, the antibiotic susceptibilities of *E. coli* in this environment appear to be negatively impacted by wastewater treatment facility effluent.**

INTRODUCTION

The ever increasing presence of antibacterial agents in household products and increased usage in hospital and agricultural practices may be leading to an increase in antimicrobial resistant bacteria (Aiello et al. 2004; Carson et al. 2008). The presence of detectable levels of antibiotics in surface waters has previously been reported by several groups (Metcalfe et al. 2003; Göbel et al. 2005; Batt et al. 2006). Batt et al. (2006) detected antibiotics such as ciprofloxacin, sulfamethoxazole, and clindamycin as far as 100 meters downstream of wastewater treatment plant effluent, with higher concentrations of these antibiotics and others at the site of wastewater treatment effluent. Metcalfe et al. (2003) found measurable levels of antibiotics in surface waters associated with domestic sewer systems. Evidence for the development of resistance in environmental bacteria due to exposure to low concentrations of antibiotics has been demonstrated. Schwartz et al. (2003) detected several antibiotic resistant bacteria and/or genes in municipal wastewater discharges. Volkmann et al. (2004) found β-lactam-resistant Enterobacteriaceae from all five municipal wastewater treatment plants that were tested. Ash and co-workers isolated antibiotic-resistant bacteria from 16 different rivers in the United States (Ash et al. 2002). Fourteen percent of the bacteria these authors isolated from the Canadian River in Oklahoma City were resistant to at least 2 antibiotics.

The purpose of this study was to evaluate the antibiotic susceptibility of *Escherichia coli* isolated from a stream in Edmond Oklahoma that receives wastewater effluent. The hypothesis is that *E. coli* isolated at or downstream of the wastewater effluent will be more resistant to antibiotics than *E. coli* isolated upstream of the wastewater effluent.

MATERIALS AND METHODS

Sample collection. Five hundred milliliter surface water samples were collected in sterile 1 liter bottles from a stream in Edmond, Oklahoma that receives effluent from wastewater treatment plants. Specifically, samples were collected approximately 100 meters upstream of two wastewater treatment plants, at the sites of wastewater effluent, and approximately 100 meters

downstream of the wastewater effluent. All samples were placed on ice and transported back to laboratory and analyzed within 24 hrs using the procedures below. Each site was sampled three times during the course of the study over a period of six months.

Isolation and identification of bacteria. Isolation and recovery of bacteria were carried out using the membrane filtration method (Eaton et al. 1995). Sample volumes of 10 ml, 1 ml, and 0.1 ml were vacuum filtered through 0.45 μm pore size filters (Pall Life Sciences, Ann Arbor, MI.). Filters were placed onto Membrane Faecal Coliform (m-FC) agar plates (Becton Dickinson, Franklin Lakes, NJ.) and incubated at 45 ^{o}C for 24 hrs. Colonies that were blue in appearance were selected and further identified by Gram staining and standard Indole, Methyl red/Vogues Proskaur, and Simmon's Citrate (IMViC) biochemical tests. Isolates identified as *Escherichia coli* were frozen in Tryptic Soy Broth (TSB) plus 30% glycerol at -80^{o}C.

Antibiotic susceptibility testing. Antibiotic susceptibility testing was conducted in accordance with the Clinical Laboratory and Standards Institute (CLSI) guidelines for antimicrobial susceptibility testing (CLSI M02-A11 2012). Bacterial suspensions were made in sterile 1x phosphate buffered saline (PBS) pH 7.4 to match a 0.5 McFarland Standard to achieve an inoculum density of approximately 1 x 10^{8} Cfu/ml. From these suspensions sterile swabs were used to inoculate the surface of Mueller-Hinton agar plates. After inoculation, antimicrobial disks were placed on the surface of the plates. Antibiotic disks (Becton Dickinson and Co., Cockeysville, MD) were the following: 10 μg ampicillin (AM-10); 20 μg amoxicillin/10 μg clavulanic acid (AmC-30); 5 μg ciprofloxacin (CIP-5); 30 μg tetracycline (TE-30); 23.75 μg sulfamethoxazole/1.25 μg trimethoprim(SXT-23.75/1.25). Plates were incubated at 35^{o}C for 18-24 hours and zones of inhibition were measured in millimeters. An isolate was designated as antibiotic resistant (AR) if it was resistant to at least one of the antibiotics tested and multiple antibiotic resistant (MAR) if it was resistant to at least two antibiotics tested. The antibiotics used in this study were chosen because of their clinical and/or agricultural significance. Each one of these antibiotics have either been found at potentially active concentrations in wastewater (Göbel et al. 2005) or have previously been associated with increased resistance in environmental *E. coli* (Parveen et al. 1997; Costanzo et al. 2005).

Statistical analysis Significant differences between the zones of inhibition for the 'upstream', 'at effluent', and 'downstream' *E. coli* isolates were determined using analysis of variance (ANOVA) with a Tukey's post hoc test.

RESULTS AND DISCUSSION

A total of 87 *E. coli* isolates were collected from a stream in Edmond, Oklahoma that receives wastewater effluent from two wastewater treatment facilities. Figure 1 shows that the mean zone of inhibition for each antibiotic tested was in general

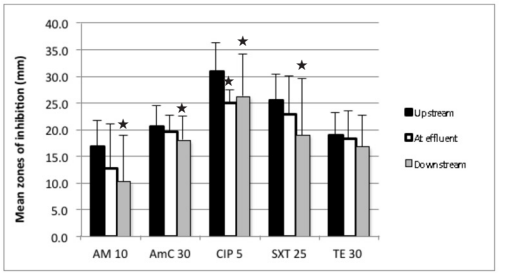

Figure 1. Antibiotic susceptibility testing for the *E. coli* isolates. The results are represented as the mean and standard deviation for all isolates tested at a given position in relation to the wastewater effluent. Asterisks indicate significant ($p < 0.05$) differences between the upstream isolates and the at effluent isolates or downstream isolates.

progressively lower at and downstream of where wastewater treatment effluent entered the stream, than upstream. For ampicillin, amoxicillin/clavulanic acid, and sulfamethoxazole/trimethoprim, the mean zones of inhibition were significantly lower ($p < 0.05$) downstream than upstream, while the mean zone of inhibition for ciprofloxacin was significantly lower ($p < 0.05$) at effluent and downstream than upstream. The prevalence of AR and MAR *E. coli* upstream was 15.8% and 7.7% respectively, compared to 36.8% and 10.5% at effluent, and 70% and 34.5% downstream (Figure 2). The prevalence of AR and MAR *E. coli* was markedly higher downstream than upstream. These results indicate that the antibiotic susceptibilities of *E. coli* in this environment are negatively impacted by effluent coming from wastewater treatment facilities,

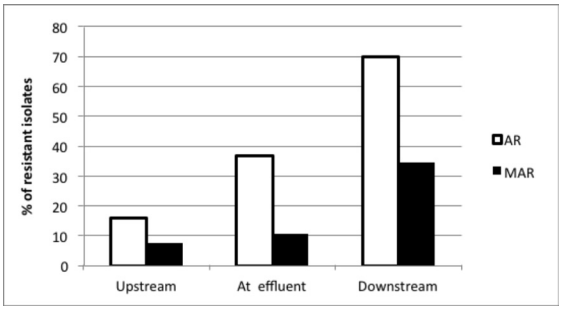

Figure 2. Prevalence of *E. coli* resistant to at least one antibiotic (AR) or at least two antibiotics (MAR).

which is similar to that of previous reports. Parveen et al. (1997) found that 94.6% of *E. coli* isolated from wastewater effluent were resistance to at least one antibiotic, which was significantly higher than 67.7% of *E. coli* isolates collected from non-effluent in the Apalachicola Bay of Florida. Watkinson et al. (2007) reported that greater numbers of antibiotic resistant bacteria were found in water adjacent to wastewater treatment discharge. The prevalence of AR and MAR *E. coli* are also similar with the findings of Servais and Passerat (2009). In their study of the Seine river watershed in France, they reported AR and MAR percentages as high as 71% and 65% respectively depending on the source (Servais and Passerat 2009). Of the antibiotics tested, ampicillin was the antibiotic with highest percentage (43.8%) of resistant isolates collected at and downstream of effluent followed by tetracycline (20.8%), sulfamethoxazole/trimethoprim (16.7%), amoxicillin/clavulanic acid (12.5%), and ciprofloxacin (4.2%) respectively (Table 1). Our finding that resistances to ampicillin, tetracycline, and sulfamethoxazole/trimethoprim were more prevalent in *E. coli* than resistances to the amoxicillin/clavulanic acid and ciprofloxacin are also consistent with previous reports (Watkinson et al. 2007; Hu et al. 2008; Servais and Passerat 2009). Resistance to the tested antibiotics may be reflective of the relative levels of these antibiotics in the effluent, which could be

Table 1. Resistance of *E. coli* isolates collected near wastewater treatment facilities to various antibiotics.

	% of resistant isolates[a]		
Antibiotic	**Upstream (n = 39)**	**At effluent (n = 19)**	**Downstream (n = 29)**
AM 10	10.2	31.6	51.7
AmC 30	7.8	5.3	17.2
CIP 5	0.0	0.0	6.9
SXT 25	2.6	10.5	20.7
TE 30	10.3	10.5	27.6

[a]Abbreviations used: AM 10, Ampicllin at 10 μg; AmC 30, Amoxicillin + Clavulanic acid at 30 μg; CIP 5, Ciprofloxacin at 5 μg; SXT 25, Sulfamethoxazole/Trimethoprim at 25 μg; TE 30, Tetracycline at 30 μg.

a bi-product of the extent to which these antibiotics are prescribed. Penicillins, tetracyclines, and sulfonamides have historically and continue to be among the most commonly prescribed antibiotics.

To the best of our knowledge, this is the first report on the impact of the wastewater treatment on the antibiotic resistance of environmental *E. coli* in central Oklahoma. A more comprehensive study involving the isolation and characterization of more isolates from additional sites around central Oklahoma would be needed to determine if this is an isolated observation or a region wide occurrence. These findings are significant because of the growing concern for the introduction of antibiotic resistant bacteria from the environment into human infections. Infections due to antibiotic resistant bacteria are more difficult to treat and are associated with increased morbidity and mortality. The ability of bacteria to share genetic material is well established. Greater numbers of antibiotic resistant bacteria in the environment due to exposure to wastewater effluent could serve as a reservoir for the horizontal spread of resistance genes to other bacteria in the environment, which could pose a health threat to individuals that might come in contact with these antibiotic resistant bacteria in these environments. Therefore, this study, along with other similar studies (Zhang et al. 2009; Huang et al. 2012), provides additional evidence for more judicial use of antimicrobials and the need to utilize more effective techniques to remove antimicrobials from wastewater.

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