REPORT

Title
Anthropogenic Influence on Tetracycline Resistance in a Rapidly Urbanizing Texas Stream

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Abstract
The objective of this study was to determine the effects of land use (agricultural and urban), environmental media (streambed sediment and water), and season (spring/summer and fall/winter) on the occurrence of tetracycline resistant bacteria and three commonly found tetracycline resistance genes (tet(O), tet(W), and tet(Q)) in a perennial stream in Texas. Water and streambed samples were collected from the perennial stream at five locations with varying landuses. Heterotrophic bacteria were enumerated on nutrient agar plates containing three different levels of tetracycline (0 mM, 0.03 mM, and 0.06 mM). Statistical analysis using SAS showed tetracycline resistant bacteria were capable of growing at both low and high tetracycline levels from samples collected from all locations regardless of season. Tetracycline resistant bacteria in streambed sediments had higher counts than resistant bacteria in water. Tetracycline resistance genes were detected throughout the year in both sediment and water from all five sampling locations. This indicates that antibiotic resistance is prevalent in this perennial stream and management practices should be implemented to decrease this problem.

Problem and Research Objectives
The ability of antibiotics to prevent disease and death has deteriorated due to the development of antibiotic resistant bacteria species (WHO, 2000 and Car et al., 2008). The development of antibiotic resistance in bacteria is mainly due to two factors: mutations of cellular DNA and acquisition of new resistance genes (Thomas and Nielsen, 2005 and Bennett, 2008). The major cause of antibiotic resistance in aquatic environments is via horizontal gene transfer along with selective pressure from the environment (Baquero et al., 2008 and Zhang et al., 2009). The selective pressure can be due to overuse or misuse of antibiotics in agricultural and human use.

The objective of this study was to determine the effects of land use (agricultural and urban), environmental media (streambed sediment and water), and season (spring/summer and fall/winter) on the occurrence of tetracycline resistant bacteria and three tetracycline resistance genes (tet(O), tet(W), and tet(Q)) in a perennial stream in Texas. These three genes were chosen because they have only recently been found in natural water environments and can be correlated with microbial communities of sewage treatments, hospital wastewater, and animal production wastewater (Chopra and Roberts, 2001, Zhang et al., 2009, and McKinney et al., 2010).

Materials/Methodology

Study Area

Carters Creek (Texas Commission of Environmental Quality (TCEQ) Segment ID: 1209C) watershed, a sub-watershed of Navasota River Basin (HUC #12070103), is located in Brazos County in East Central Texas. It covers about 146 square kilometers running through the Southern Post Oak Savanna ecoregion. The 27 kilometers long perennial stream passes through landuse that is heavily urbanized in the upper reach of the watershed where it originates in Bryan/College Station, and becomes more rural in the lower reach.. Average annual rainfall in the watershed is 165 mm and average winter and summer temperature is 15°C and 24°C. The average respective stream flow during summer and winter is 115 L/d.

Sampling Protocol

Water and sediment samples were collected at five sampling locations along Carters Creek. The five sampling locations are differentiated by landuse shown in Figure 1. Samples were collected five times during the spring/summer season (between March and August) and five times during the fall/winter season (between September and February). Approximately 200 mL of water was collected using sterile Whirlpak® bags. Samples from the water column were collected from below the surface of the water by manually dipping the sample bag into the creek. Approximately 100 g of the upper sediments (about 5 cm) were collected using a shovel and stored in Whirlpak® bags. The samples were stored in coolers with Blueice® (at 5°C) and transported to laboratory. Fifty grams of the sediment samples were stored at -80°C for molecular analysis. One hundred milliliters of water samples were filtered on 0.45 µm filters then stored at -80°C for molecular analysis. The remaining water and sediment samples were processed within 8 h for viable culturing.

Culture Based Methods: Enumerating Antibiotic Resistant Bacteria

Tenfold serial dilutions were prepared with 10^{-1} dilution defined as one g of sediment sample diluted in 9 mL of sterilized water or 1 mL of water sample in 9 mL sterilized water. The diluted samples were spread plated onto 100 × 10 mm culture plates containing nutrient agar (Difco©, MD) and different levels of tetracycline hydrochloride (Calbi Chem, CA) (0 mM, 0.03 mM, and 0.06 mM . Studies have shown tetracyclines are lethal to 70% of bacteria from sandy soils at a concentration of 10 mg/L (Sarmah et al., 2006). These high concentrations will ensure that the cultural bacteria evaluated are truly resistant. The plates were incubated at 35°C for 48 h to ensure the inclusion of slow-growing heterotrophic bacteria, and shielded from light to prevent photodegradation of tetracycline.

Molecular Methods: Amplifying ARGs

DNA extraction

Genomic DNA was extracted from one gram sediment from each sampling location using MoBio© Ultra clean Soil DNA extraction kit (MoBio, CA) according to the manufacturer's protocol. Genomic DNA from the water samples was extracted from the filters using the MoBio© Ultra Clean Water DNA kit according to the manufacturer's protocol.

Qualitative PCR

A qualitative PCR assay was performed in order to determine which of the three tetracycline ARGs were present at each sampling location. Forward and Reverse primers described by Aminov et al. (2001) were used. Promega© PCR Master Mix (Promega, WI) which included buffer, dNTPs, and Taq DNA polymerase were used for the PCR. The temperature program consisted of initial denaturing at 95°C followed by 30 cycles of 15s at 95°C, 30s at the annealing temperature (*tet*(O) 60°C, *tet*(W) 64°C, *tet*(Q) 63°C, and 16S RNA 50°C), and a final extension of 7 min at 72°C. The PCR product was then separated by gel electrophoresis using 1% agarose at 5 V/cm. The gel was documented using Photodine® gel documentation station. Presence of a band was considered to confirm the presence of a targeted gene. If a band was not detected, the sample was spiked with a positive control for the gene and underwent a new PCR reaction under the previous conditions to determine if PCR inhibitors were present.

Statistical Analysis

Statistical Analysis was performed using SAS 9.2(SAS Institute Inc., Cary, NC). Significance of the data was defined as p-values ≤ 0.05. One-way ANOVA (Proc GLM) was used to generate population means, standard deviation, 95% confidence intervals (CI), and significant difference. The least significant difference (LSD) test within Proc GLM was also used for comparison of population means for categorizing significant groups

Principal Findings

Tetracycline resistant bacteria were capable of growing under all conditions year around indicating the occurrence and prevalence of antibiotic resistance in this study area. This finding was further supported by the presence of tetracycline resistant genes at all sampling locations in both sediment and water regardless of season. It was also observed that samples taken from streambed sediments had significantly higher resistant bacteria counts than water samples. Streambed sediments may promote the maintenance of resistant bacteria populations better than water and could be a potential reservoir of ARGs. There was no seasonal variability on the bacteria counts. There was no spatial variability for tetracycline resistant bacteria, and inputs from wastewater treatment facilities did not influence the tetracycline resistant bacteria counts. The rapidly urbanizing watershed used in this study may have prevented significant changes in landuse creating a more uniformed watershed and explains the lack of significant differences between sights.

Significance

Results from this research indicate that tetracycline resistant bacteria are prevalent in this watershed. Further research is being conducted to characterize the resistance in this watershed to determine if the resistance is a potential health concern.

References Cited

Aminov, R.I., Garrigues-Jeanjean, N., and Mackie, R.I. 2001. Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection protein. Appl. Environ. Microbiol. 67 (1), 22-32.

Baquero, F., Martinez, J., and Canton, R. 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19(3), 260-265.

Bennet, P.M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Brr J. Pharmacol. 152, S347-S357.

Car, O., Högberg, L.D, Murray, M., Nordberg, O., Sivaraman, S., Lundborg, C.S., Do, A.D., and Tomson, G. 2008. Meeting the challenge of antibiotic resistance. BMJ. 337 (a1438), 726-728.

Chopra, I.and Roberts, M. 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65 (2) 232-260.

McKinney, C.W., Loftin, K.A., Meyer, M.T., Davis, J.G., and Pruden, A. 2010. *Tet* and *sul* Antibiotic Resistance Genes in Livestock Lagoons of Various Operation Type, Configuration, and Antibiotic Occurrence. Environ. Sci. Technol. In Press.

Sarmah, A.K., Meyer, M.T., and Boxall, A.B.A. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (Vas) in the environment. Chemosphere. 65, 725-759.

Thomas, C.M. and Nielsen, K.M. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Micro. 9(3), 711-721.

World Health Organization (WHO). 2000. WHO Annual Report on Infectious Disease: Overcoming Antimicrobial Resistance; World Health Organization: Geneva, Switzerland. Available at: http://www.who.int/infectious-disease-report/2000/. Accessed 29 Nov 29 2008.

Zhang, X., Zhang, T., and Fang, H.H. 2009. Antibiotic resistance genes in water environment. Appl. Microbiol. Biotechnol. 82(3), 397-414

PUBLICATION

No reports were published during the reporting period.

NOTABLE AWARDS AND ACHIEVEMENTS

No awards were received during the reporting period.