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Microbial source tracking in shellfish harvesting waters in the Gulf of Nicoya, Costa Rica

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ABSTRACT

Current microbial water quality monitoring is generally limited to culture-based measurements of fecal indicator bacteria (FIB). Given the many possible sources of fecal pollution within a watershed and extra-intestinal FIB reservoirs, it is important to determine source(s) of fecal pollution as a means to improve water quality and protect public health. The principal objective of this investigation was to characterize the microbial water quality of shellfish harvesting areas in the Gulf of Nicoya, Costa Rica during 2015. In order to achieve this objective, the specificity and sensitivity of 11 existing microbial source tracking (MST) PCR assays, associated with cows (BacCow), dogs (BacCan, DogBac), domestic wastewater (PMMoV), general avian (GFD), gulls (Gull2), horses (HorseBac, HoF), humans (HF183, HPyV), and pigs (PF), were evaluated using domestic wastewater and animal fecal samples collected from the region. The sensitivity of animal-associated assays ranged from 13 to 100%, while assay specificity ranged from 38 to 100%. The specificity of pepper mild mottle virus (PMMoV) and human polyomavirus (HPyV) was 100% for domestic wastewater, as compared to 94% specificity of the HF183 *Bacteroidales* marker. PMMoV was identified as a useful domestic wastewater-associated marker, with concentrations as high as 1.1×10^5 copies/ml and 100% sensitivity and specificity. Monthly surface water samples collected from four shellfish harvesting areas were analyzed using culture-based methods for *Escherichia coli* as well as molecular methods for FIB and a suite of MST markers, which were selected for their specificity in the region. While culturable *E. coli* results suggested possible fecal pollution during the monitoring period, the absence of human/domestic wastewater-associated markers and low FIB concentrations determined using molecular methods indicated sufficient microbial water quality for shellfish harvesting. This is the first study to our knowledge to test the performance of MST markers in Costa Rica as well as in Central America. Given the lack of wastewater treatment and the presence of secondary sources of FIB, this study highlights the importance of an MST toolbox approach to characterize water quality in tropical regions. Furthermore, it confirms and extends the geographic range of PMMoV as an effective tool for monitoring domestic wastewater pollution.

1. Introduction

In Costa Rica, it is estimated that only 5% of domestic wastewater is treated prior to surface water discharge (Contraloría de la República, 2013). Between 70 and 80% of wastewater contamination reaching the Pacific Coast via the Gulf of Nicoya, the largest estuary in Costa Rica, stems from rivers surrounding the densely-populated San José metropolitan area (Mora Alvarado et al.,

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2012). The Gulf of Nicoya, a medium-sized, tropical (dry and rainy seasons) estuary, is one of the most important fishing areas in the country and home to various community fishing associations (Araya et al., 2007; Chacón et al., 2007). The Gulf of Nicoya contains areas that have been formally designated as marine responsible fishing areas by the Costa Rican government – these are areas in which fisherman catch fish and shrimp as well as cultivate oysters using responsible practices. Since approximately 30% of Costa Rica's domestic seafood comes from the Gulf of Nicoya (INCOPESCA, 2013), domestic wastewater pollution threatens the sustainable use of this coastal ecosystem as well as the local and national economy.

The microbial water quality of the Gulf of Nicoya is influenced by a mixture of human and non-human sources that enter the Gulf in both the upper (shallow, less than 20 m) and lower (deeper, ranging from 25 to 100 m in depth at the mouth) regions. The upper region is influenced by the second largest watershed in Costa Rica, Greater Tempisque River Watershed (Palter et al., 2007), which contributes high concentrations of fecal coliforms (as much as 2,821 colony forming units (cfu)/100 ml) from the surface wastewater discharge of several cities (Mora Alvarado, 2004) as well as thousands of commercial farms that raise chicken, cattle, pigs, sheep, and goats (CR SENASA, 2012). The lower region of the Gulf of Nicoya is influenced by the poor water quality of the Barranca River (geometric mean fecal coliforms 2,150 cfu/100 ml) and the Grand River of Tarcóles (geometric mean fecal coliforms 840,000 cfu/100 ml), which has the highest concentration of fecal coliforms in comparison to other rivers and receives a large portion of the wastewater generated in the San José metropolitan area (Mora Alvarado, 2004). Wild and domesticated animals also contribute to the fecal pollution in the Gulf of Nicoya and principally include large quantities of seabirds and migratory birds, horses, livestock, and dogs. To the best of our knowledge, the contribution of each of these sources of fecal pollution over time has yet to be investigated.

Currently, the Costa Rican National Water Laboratory monitors recreational surface water quality for the presence of fecal indicator bacteria (FIB; e.g. fecal coliforms) using culture-based techniques. While no legislation exists regarding the microbial water quality of shellfish harvesting areas (Corrales and Vindas, 2014), the maximum allowed fecal coliform concentrations for fish and shrimp aquaculture are 1,000 cfu/100 ml and 100 cfu/100 ml, respectively (Mora Alvarado, 2004). Although the Costa Rican governmental water quality monitoring utilizes fecal coliforms as an indicator, it has been shown that *Escherichia coli* is a more effective indicator of recent contamination as well as more representative of warm-blooded animal feces (WHO, 2009). A previous study demonstrated that *E. coli* concentrations varied over time in two shellfish harvesting areas within the Gulf of Nicoya, with noticeable differences between dry and rainy seasons (Corrales and Vindas, 2014); however, mean *E. coli* concentrations were still within the bounds of the approved category requiring no depuration or relaying (i.e., $\leq$230 most probable number (MPN)/100 g per European Union Shellfish Harvesting Criteria and $\leq$43 MPN/100 ml per the United States of America (USA) National Shellfish Sanitation Program; Corrales and Vindas, 2014; Lee et al., 2008; Oliveira et al., 2011).

While all types of fecal pollution present a risk to human health, it has been demonstrated that pig, gull, and chicken feces pose less of a health risk to humans in comparison to cow and human feces (Soller et al., 2010). Even though fecal coliforms, *E. coli*, and enterococci are the most commonly used FIB for monitoring microbial water quality in marine systems, they cannot be used to distinguish between human and animal sources nor does their presence necessarily indicate active fecal contamination given the possibility of re-suspension from sediments (Anderson et al., 2005; Davies et al., 1995; Kelsey et al., 2004; Liang et al., 2015). Furthermore, it has been demonstrated that secondary reservoirs of *E. coli* exist in tropical environments (e.g. soil and runoff; *reviewed in* Winfield and Groisman, 2003) and genomic analyses have demonstrated the existence of environmentally-adapted strains of *E. coli* that are indiscernible from commensal *E. coli* using traditional culture-based assays (Luo et al., 2011). Accordingly, a microbial source tracking (MST) toolbox approach that employs multiple markers representing different sources is preferred to circumvent the aforementioned difficulties with FIB and to accurately characterize the extent of fecal pollution (Harwood et al., 2013b; Liang et al., 2015; Stoeckel and Harwood, 2007). While the establishment of MST markers has occurred primarily in non-tropical, high-income countries to date, the usefulness of this approach in achieving the United Nations' Sustainable Development Goals in all countries was recently highlighted (UN Water, 2015).

The principal objective of this investigation was to determine the surface water quality of four responsible fishing areas within the Gulf of Nicoya as well as the sources of fecal pollution contributing to any impairment during the 2015 wet and dry seasons. To achieve this objective, the sensitivity (i.e., the percentage of target host fecal samples that yield true positive results) and specificity (i.e., the percentage of non-target host fecal samples that yield true negative results) of MST markers for cows, dogs, birds, horses, pigs, and domestic wastewater were determined through the analysis of animal fecal samples as well as domestic wastewater collected from the region. To the best of our knowledge, this is the first study to identify MST markers for use in Costa Rica and the first study of its kind in Central America. The surface water quality of four shellfish harvesting areas was then analyzed using *E. coli* MPN methods as well as molecular methods for a suite of FIB and domestic wastewater/human-associated MST markers, which were selected for their suitability in the region.

2. Materials and methods

2.1. Feces and wastewater collection, preservation, and nucleic acid purification

Animal feces and domestic wastewater samples were collected using standard, sterile techniques from areas within the Gulf of Nicoya drainage basin. Fecal samples were collected from the following animals identified as most abundant in the region (CR SENASA, 2012): chickens ($n = 8$), cows ($n = 8$), pigs ($n = 7$), dogs ($n = 8$), horses ($n = 8$), and sea birds ($n = 8$). Samples were maintained at 4 °C and nucleic acid purification was initiated within 24 h of collection. A wet weight of 0.25 g of each fecal sample was preserved in bead beating tubes from the MoBio PowerViral™ Environmental RNA/DNA kit (MoBio Laboratories, Inc.; Carlsbad, CA, USA) with 0.5 vol of lysis buffer and stored at −20 °C. Upon arrival to the University of South Florida, samples were thawed and nucleic acid purification was resumed by adding the remaining 0.5 vol of lysis buffer and then following manufacturer's instructions. The First Strand Synthesis Superscript III Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA), with random hexamer primers, was used to synthesize cDNA. One extraction blank was processed alongside fecal samples and was negative for all MST marker PCR assays.

Eight 100-ml untreated, domestic wastewater samples were collected, approximately each month from May to December 2015, in sterile Whirl-Pak® Bags (Nasco, Fort Atkinson, WI, USA) from the domestic wastewater treatment facility in El Roble, Puntarenas. In addition to receiving treated industrial wastewater, this facility serves an estimated 29,847 permanent habitants, as well as a local