Water Research 88 (2016) 613–622

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Utility of *Helicobacter* spp. associated GFD markers for detecting avian fecal pollution in natural waters of two continents

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ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form
18 October 2015
Accepted 26 October 2015
Available online 9 November 2015

Keywords:
Microbial source tracking
Fecal indicator bacteria
Avian fecal pollution
Molecular markers
Wastewater
Quantitative PCR

ABSTRACT

Avian fecal droppings may negatively impact environmental water quality due to the presence of high concentrations of fecal indicator bacteria (FIB) and zoonotic pathogens. This study was aimed at evaluating the performance characteristics and utility of a *Helicobacter* spp. associated GFD marker by screening 265 fecal and wastewater samples from a range of avian and non-avian host groups from two continents (Brisbane, Australia and Florida, USA). The host-prevalence and -specificity of this marker among fecal and wastewater samples tested from Brisbane were 0.58 and 0.94 (maximum value of 1.00). These values for the Florida fecal samples were 0.30 (host-prevalence) and 1.00 (host-specificity). The concentrations of the GFD markers in avian and non-avian fecal nucleic acid samples were measured at a test concentration of 10 ng of nucleic acid at Brisbane and Florida laboratories using the quantitative PCR (qPCR) assay. The mean concentrations of the GFD marker in avian fecal nucleic acid samples (5.2×10^3 gene copies) were two orders of magnitude higher than non-avian fecal nucleic acid samples (8.6×10^1 gene copies). The utility of this marker was evaluated by testing water samples from the Brisbane River, Brisbane and a freshwater creek in Florida. Among the 18 water samples tested from the Brisbane River, 83% ($n = 18$) were positive for the GFD marker, and the concentrations ranged from 6.0×10^1–3.2×10^2 gene copies per 100 mL water. In all, 92% ($n = 25$) water samples from the freshwater creek in Florida were also positive for the GFD marker with concentrations ranging from 2.8×10^1–1.3×10^4 gene copies per 100 mL water. Based on the results, it can be concluded that the GFD marker is highly specific to avian host groups, and could be used as a reliable marker to detect the presence and amount of avian fecal pollution in environmental waters.

1. Introduction

Microbial source tracking (MST) is a process of determining the sources of fecal pollution in waters. A range of MST tools have been developed to identify sewage and animal-derived fecal pollution in global waters. The initially developed MST methods were library-dependent, requiring fingerprint matching of fecal indicator bacteria (FIB) from different animals (host groups) to compare with patterns of FIB isolated from environmental waters (Scott et al., 2002; Stoeckel and Harwood, 2007). Library-dependent methods can be costly and time consuming due to the requirement for developing a representative library of isolates from host groups and environmental waters. In addition, the performance of a library is influenced by several factors such as geographical stability, temporal stability, and complexity in statistical analysis (Field and Samadpour, 2007; Gordon et al., 2002; Hartel et al., 2002; Harwood et al., 2003).

In contrast, another set of methods known as "library-independent methods" primarily involve identifying a specific DNA sequence or target gene (known as molecular marker) of a bacterial species or virus in host groups, and identifying the same marker in environmental water samples using polymerase chain reaction (PCR) assays. Many PCR and quantitative PCR (qPCR) based assays have been developed to identify (and in some cases quantify) a

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http://dx.doi.org/10.1016/j.watres.2015.10.050
0043-1354/

wide array of molecular markers in host groups and environmental waters (Ahmed et al., 2008, 2010b; Harwood et al., 2014). The application of these markers for MST studies depends on several performance characteristics such as host-specificity, host-prevalence, evenness, persistence, and relevance to health risks (Harwood et al., 2014; Stoeckel and Harwood, 2007). It has been recommended that the performance characteristics of a newly developed marker(s) must be assessed prior to field application in new geographical areas (Stoeckel and Harwood, 2007; US EPA, 2005).

Fecal pollution from avian wildlife may impact the microbiological quality of environmental waters (Converse et al., 2012; Edge and Hill, 2007; Lee et al., 2013; Lévesque et al., 1993; Lu et al., 2008; Shibata et al., 2010; Wither et al., 2005). Avian waste is known to contain high concentrations of FIB (*Escherichia coli* or *Enterococcus* spp.) (Alderisio and DeLuca, 1999; Fogarty et al., 2003; Ge et al., 2010). For example, *E. coli* concentrations in gull feces may range from 10^5–10^9 per g of feces (Fogarty et al., 2003). In addition, the presence of pathogenic *E. coli* (Ahmed et al., 2012; Wallace et al., 1997), *Campylobacter* spp. (Fallacara et al., 2004; Kinzelman et al., 2008), *Salmonella* spp. (Fallacara et al., 2004; Kinzelman et al., 2008), *Giardia lamblia* (Kuhn et al., 2002), *Cryptosporidium parvum* (Kuhn et al., 2002), and antibiotic resistance genes (Cizek et al., 2007; Middleton and Ambrose, 2005; Simões et al., 2010) have been reported in avian feces. Humans can come in contact with avian waste by recreational activities or consuming shellfish contaminated with pathogenic microorganism from different avian species. Avian species are highly mobile and display varied feeding habits. Therefore, they can be considered important vehicles for spreading pathogens that may present significant human health risks.

A recent study has reported the development of two qPCR assays for the identification of gull, Canada goose, duck and chicken feces-associated genetic markers in various host groups in the United States, Canada and New Zealand (Green et al., 2012). For the identification of host-specific unique fecal sequences, the authors used microplate subtractive hybridization, which is able to differentiate between very closely related hosts, and between hosts that live in close contact. Based on the 16S rRNA gene fragments, the authors identified *Catellicoccus marimammalium* associated GFC and unclassified *Helicobacter* spp. -associated GFD markers. The distributions of these markers in avian feces across USA, Canada and New Zealand suggested they might have broad applicability for MST studies in other parts of the world (Green et al., 2012).

The primary aim of this study was to evaluate the host-specificity and -prevalence of avian feces-associated GFD marker by testing fecal samples from a variety of avian and non-avian host groups at the Commonwealth Industrial Scientific Research Organization (CSIRO) laboratory in Brisbane, Australia, and the University of South Florida (USF), Florida, USA. Environmental water samples (potentially affected with avian feces) were collected from the Brisbane River (Brisbane) and a freshwater creek (Florida). All environmental water samples were tested for the presence of the GFD marker using a qPCR assay along with FIB enumeration using culture-based methods. The host-specificity and -prevalence of the GFD marker along with their presence in environmental waters were then used to validate the presence of avian fecal pollution in the studied water bodies.

2. Materials and methods

2.1. Avian and non-avian host group sampling

To determine the host-specificity and -prevalence of the avian feces-associated GFD markers, individual and composite fecal and wastewater samples were collected from various avian and non-avian host groups in Brisbane and Florida (Table 1). Additional information on the fecal and wastewater samples is given in the Supplementary note 1. All samples were transported on ice to the respective laboratories, stored at 4 °C, and processed within 6–24 h.

2.2. Concentration of cattle, human and pig wastewater samples

The wastewater samples collected from host groups in Brisbane were concentrated with Amicon® Ultra-15 (30 K) Centrifugal Filter Devices (Merck Millipore Ltd.). In brief, 10 mL of wastewater sample was added to the Amicon device, and centrifuged at 4,750 *g* for 10 min. Entire volumes (180–200 μL) of concentrated samples were collected from the filter device sample reservoir using a pipette (Ahmed et al., 2010a). The concentrated samples were stored at −20 °C for a maximum of 24 h prior to nucleic acid extraction.

2.3. Environmental water sampling in Brisbane and Florida

Water samples were collected from six sites on one occasion in December 2013 (designated B1 to B6) along the Brisbane River, Brisbane, Australia. Each site was sampled in triplicate giving a total number of 18 water samples. From each site, grab water samples were collected in 10 L sterile containers from 30 cm below the water surface, and transported on ice to the laboratory where they were processed within 6 h of collection. Site B1 is located upstream of the Brisbane Central Business District (CBD). This site receives overflow of water from the Wivenhoe Reservoir. The suspected sources of fecal pollution at this site include waterfowl and wildlife. Local residents also use this site for swimming and fishing. Site B2 is located in a peri-urban, non-sewered catchment feeding into the Brisbane River. The potential sources of pollution at this site include cattle, horses, septic systems and wildlife. Site B3 is a major tributary of the Brisbane River and is tidally influenced. The catchment where the site B3 is located has residential and industrial developments, and is serviced by a wastewater treatment plant (WWTP) which discharges treated wastewater into the Brisbane River. The elevated levels of FIB in this site have been a major water quality issue identified by the local catchment water quality-monitoring program. Site B4 is located in a highly urban area and is also tidally influenced. This site receives urban runoff through a stormwater drain. Sites B5 and B6 are located downstream of the CBD in highly urbanized areas. Potential sources of fecal pollution at these sites include waterfowl and recreational boats. Sampling sites, their location, suspected sources of fecal pollution and physico-chemical water quality parameters have been provided in the Supplementary Table 1.

Water samples in Florida were collected monthly from five sites (designated F1 to F5) for five consecutive months in a freshwater creek in Kissimmee, Florida, USA. From each site, grab water samples were collected in 1 L sterile containers from 30 cm below the water surface, and transported on ice to the laboratory and processed within 6–8 h.

The watershed is a channelized wetland area managed for wildlife, with frequent regulatory exceedances of water quality criteria for FIB. The area is rich in waterfowl and other wildlife, and adjacent to some suburban development though there are no known point sources of sewage contamination. Samples were collected mid-channel from a research boat. Sampling sites, suspected sources of fecal pollution and physico-chemical water quality parameters have been provided in the Supplementary Table 2.