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Validation of *Bacteroidales* quantitative PCR assays targeting human and animal fecal contamination in the public and domestic domains in India

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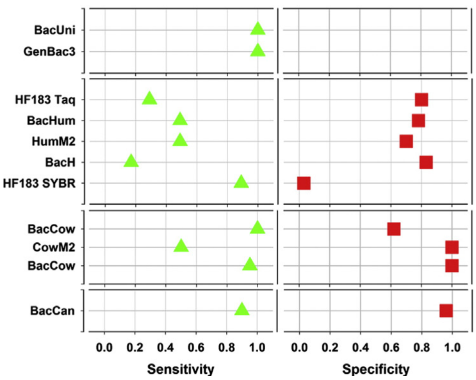
HIGHLIGHTS

- First report of *Bacteroidales* qPCR assay validation in India
- Ten qPCR assays evaluated with fecal samples from human and animal sources in India
- BacHum best of five human-associated assays tested on Indian fecal samples
- BacUni, BacHum, HumM2, BacCan and BacCow recommended for future MST use in India

GRAPHICAL ABSTRACT



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ABSTRACT

We compared host-associated *Bacteroidales* qPCR assays developed in the continental United States and Europe for the purpose of measuring the effect of improved sanitation on human fecal exposure in rural Indian communities where both human and animal fecal loading are high. Ten candidate *Bacteroidales* qPCR assays were tested against fecal samples (human, sewage, cow, buffalo, goat, sheep, dog and chicken) from a test set of 30 individual human, 5 sewage, and 60 pooled animal samples collected in coastal Odisha, India. The two universal/general *Bacteroidales* assays tested (BacUni, GenBac3) performed equally well, achieving 100% sensitivity on the test set. Across the five human-associated assays tested (HF183 Taqman, BacHum, HumM2, BacH, HF183 SYBR), we found low sensitivity (17 to 49%) except for HF183 SYBR (89%), and moderate to high cross-reactivity with dog (20 to 80%) and chicken fecal samples (60 to 100%). BacHum had the highest accuracy (67%), amplified all sewage samples within the range of quantification (ROQ), and did not cross-react with any fecal samples from cows, the most populous livestock animal in India. Of the ruminant- and cattle-associated assays tested (BacCow, CowM2), BacCow was more sensitive in detecting the full range of common Indian livestock animal fecal sources, while CowM2 only detected cow sources with 50% sensitivity. Neither assay cross-reacted with human sources.

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BacCan, the dog-associated assay tested, showed no cross-reactivity with human sources, and high sensitivity (90%) for dog fecal samples. Overall, our results indicate BacUni, BacHum, HumM2, BacCan and BacCow would be the most suitable MST assays to distinguish and quantify relative amounts of human-associated and livestock/domestic animal-associated contributions to fecal contamination in Odisha, India.

1. Introduction

Microbial contamination from human fecal matter is a leading cause of preventable infectious disease, disability, and death in communities lacking sanitation and good hygiene (Wagner and Lanoix, 1958). In India, where 626 million mostly rural people practice open defecation (WHO/UNICEF, 2012) and 535,000 children under age five die each year from diarrhea (Boschi-Pinto et al., 2008), the situation is of particular concern. Adequate human sanitation is clearly necessary to prevent environmental contamination and reduce exposure to diarrhea and other excreta-related human pathogens, for example, soil-transmitted helminths. Yet questions remain about the effectiveness of simple onsite household sanitation facilities, such as pit latrines, in reducing contamination and diarrhea diseases on their own, as well as the level of community coverage necessary to achieve impacts (Clasen et al., 2012).

As part of a large study in Coastal Odisha, India on the effectiveness of providing household latrines to reduce diarrhea and helminth infections in rural communities (Clasen et al., 2012), changes in human fecal contamination and exposure in study communities are being examined. To assess exposures, fecal–oral pathways need to be considered in both the public domain (for example, local surface and groundwater sources located in public places and used for drinking, washing, and bathing by a number of people in the community) and domestic domain (for example, on hands and in stored drinking water in the home usually under the control of a household) of disease transmission, for which contamination sources and risks may differ (Cairncross et al., 1996). Furthermore, as is true across rural India, households in study communities own livestock with which they share public and private spaces and animal sanitation is poor. Cattle are among the most commonly owned species, unsurprising given their important role as a source of nutrition, fuel, transport and wealth for many rural Indian households (Tipathy, 2001). Traditional fecal indicator bacteria (FIB) such as total coliforms, thermotolerant coliforms, *Escherichia coli*, and members of the genus *Enterococcus* (the enterococci), are widely used to assess fecal contamination in the environment, but originate from both humans and animals (Leclerc et al., 2001). Thus, in order to assess changes in human fecal exposure attributable to improved human sanitation and understand its health impacts in communities with poor animal sanitation, new tools are needed that can distinguish human from non-human animal fecal contamination in study settings.

Microbial source tracking (MST) is an emerging approach to discriminate and quantify human and other animal fecal contamination sources in the environment. Among a variety of proposed MST techniques, host-associated *Bacteroidales* genetic markers are increasingly used as a complement or alternative to standard FIB in the developed world. Members of the order *Bacteroidales* are strictly anaerobic and highly abundant bacteria in human and other animal intestines and feces (Paster et al., 1994). Furthermore, *Bacteroidales* populations adapt to their hosts differently, allowing identification of host sources of fecal contamination using host-associated genetic markers (Bernhard and Field, 2000a,b). Recent studies have shown the relevance of MST using *Bacteroidales* genetic markers to identify fecal sources and assist in targeting public health interventions in less developed countries, including Kenya (Jenkins et al., 2009), Tanzania (Pickering et al., 2011, 2012; Mattioli et al., 2012) and Bangladesh (Ahmed et al., 2010; Ferguson et al., 2012; Knappett et al., 2011). To our knowledge, however, no MST *Bacteroidales* assays have been applied to address fecal pollution problems in India. Recently, performance of several human and non-human targeting MST assays was thoroughly assessed in a large-scale multi-laboratory MST method comparison study (Boehm et al., 2013). Yet applicability of the results to other regions and countries is potentially limited as all fecal samples were collected in California (Boehm et al., 2013). Because geographical differences significantly affect sensitivity and specificity of host-associated *Bacteroidales* assays (Gawler et al., 2007; Balleste et al., 2010; Ahmed et al., 2009; Jenkins et al., 2009; Tambalo et al., 2012b; Reischer et al., 2013), performance assessment is necessary prior to application of these assays in a new region of interest such as India.

The primary objectives of this research were, therefore, (1) to evaluate the performance of candidate assays for application in India, in terms of sensitivity and specificity to distinguish human and major animal fecal contamination, by testing the assays against fecal samples of known origin collected in Coastal Odisha, and (2) to identify the best performing host-associated MST assays, based on testing results, for large-scale application to evaluate sanitation impacts in Odisha, India. The secondary objective was to explore potential variability in MST assay performance on feces from healthy humans versus patients with diarrhea.

2. Materials and methods

2.1. Study area overview

This study was conducted in rural and urban areas of Puri and Khorda districts in Coastal Odisha, along the Bay of Bengal, including Bhubaneswar, Odisha's state capital (Fig. 1). Study area populations have access to improved drinking water sources from public deep and private shallow tube wells or to municipal water systems. However, access to improved sanitation is poor, resulting in large segments practicing open defecation, while open ponds continue to be used in rural areas for bathing, washing, and anal cleansing after open defecation. Over 80% of rural Odisha households own livestock, with populations highest for cows, followed by goats, sheep, and buffalos (Government of Odisha, 2013). Pigs are rare, accounting for less than 2.5% of the total livestock population, while poultry are relatively common. In addition to livestock animals, free roaming domestic dogs can be observed in large numbers in rural and urban communities in Odisha.

2.2. Identification of candidate *Bacteroidales* qPCR assays

A candidate set of ten host-associated assays, comprising two universal, five human, two cows, and one dog, was targeted for identification from the literature, as the first step toward selection of a validated optimal subset for application in the Odisha sanitation study.

Only one previous study testing the human-associated *Bacteroidales* qPCR assay HF183 SYBR has been reported in South Asia, in Dhaka, Bangladesh, reporting 87% sensitivity and 93% specificity (Ahmed et al., 2010). Thus, HF183 SYBR was identified as one of the five candidate human-associated assays. We further searched the peer-reviewed literature to identify assays performing well in developed and/or less developed countries. One problem, however, was that assay performance varied considerably from one study to the next because of different performance evaluation criteria, use of a small number of test samples, and potential influence of inter-laboratory variability, making it difficult to assess and compare performance and geographical stability across studies. Recently, a comprehensive large scale MST assay evaluation was completed in California (Boehm et al., 2013). The study assessed