JOURNAL OF ENVIRONMENTAL SCIENCES XX (2017) XXX–XXX

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES www.jesc.ac.cn

Quantification of hookworm ova from wastewater matrices using quantitative PCR

Pradip Gyawali[1,2,*], Warish Ahmed[1], Jatinder Sidhu[1,2], Paul Jagals[2], Simon Toze[1,2]

1. CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Qld 4102, Australia
2. School of Public Health, The University of Queensland, Herston Road, Qld 4006, Australia

ARTICLE INFO

Article history:
Received 22 April 2016
Revised 20 September 2016
Accepted 26 October 2016
Available online xxxx

Keywords:
Wastewater matrices
Quantitative PCR
Hookworm ova
Health risks

ABSTRACT

A quantitative PCR (qPCR) assay was used to quantify *Ancylostoma caninum* ova seeded into treated wastewater and unseeded wastewater and sludge samples. We have estimated the average gene copy numbers for a single ovum using a mixed population of ova. The average gene copy numbers derived from the mixed population were used to estimate numbers of hookworm ova in *A. caninum* seeded and unseeded wastewater and sludge samples. The newly developed qPCR assay estimated an average of 3.7×10^3 gene copies per ovum, which was then validated by seeding known numbers of hookworm ova into treated wastewater. The qPCR estimated an average of (1.1 ± 0.1), (8.6 ± 2.9) and (67.3 ± 10.4) ova for treated wastewater that was seeded with (1 ± 0), (10 ± 2) and (100 ± 21) ova, respectively. The further application of the qPCR assay for the quantification of *A. caninum* ova in unseeded wastewater matrices indicated that 50%, 90% and 67% of treated wastewater (1 L), raw wastewater (1 L) and sludge (~4 g) samples had variable numbers of *A. caninum*gene copies present. After conversion of the qPCR estimated gene copy numbers to ova numbers for unseeded wastewater samples treated wastewater, raw wastewater, and sludge samples had an average of 0.02, 1.24 and 67 ova, respectively. The result of this study indicated that qPCR can be used for the quantification of hookworm ova from wastewater and sludge samples; however, caution is advised in interpreting qPCR generated data for health risk assessment because of variable numbers of gene copies in an ovum depending on the cell development stage of an ovum.

Introduction

Wastewater use is expected to increase significantly to meet rapidly growing demands for potable and non-potable water supplies due to growing population and changing the climate (Hanjra et al., 2012; International Water Association, 2008; The World Bank, 2010). It has been estimated that around 20 million ha of agricultural lands are irrigated with treated as well as raw wastewater (Carr, 2005; International Water Association, 2008). In addition, direct application of raw wastewater into agricultural land is a common practice in developing countries (Trang et al., 2006; Vuong et al., 2007). This practice has serious public health implications due to the presence of high numbers of enteric pathogens and their potential transmission to humans via agricultural products (Jimenez et al., 2007; Toze, 2006).

The extent of the health risk, however, depends on several factors such as numbers of pathogens present in wastewater, infective dose, exposure routes and the susceptibility of the exposed individual (Haas et al., 1999; Navarro and Jimenez, 2011). Among the disease-causing microbial pathogens in wastewater, helminths such as hookworm ova/larvae pose a

* *Corresponding author*. E-mail: pradep033@yahoo.com (Pradip Gyawali).

http://dx.doi.org/10.1016/j.jes.2016.10.019
1001-0742/

Please cite this article as: Gyawali, P., et al., Quantification of hookworm ova from wastewater matrices using quantitative PCR, J. Environ. Sci. (2017), http://dx.doi.org/10.1016/j.jes.2016.10.019

significant health risk to humans because as low as <10 viable ova/larvae can cause infections in humans (Abaidoo et al., 2010; WHO, 2012).

Wastewater treatment processes are designed to inactivate the viable hookworm ova and consequently minimise the potential health risks associated with the use of treated wastewater (US EPA, 2003; WHO, 2006). However, complete inactivation of hookworm ova from wastewater and sludge (liquid and solid) is often difficult to achieve in developing countries due to the lack of ignorance and technology required for treatment (Konate et al., 2012; Konate et al., 2013; Toze, 2006). Hookworm ova can remain viable in the environment for up to a year under favourable conditions (Gyawali et al., 2015a). Therefore, a better understanding of the health risk posed by hookworm ova in the wastewater matrices (liquid and sludge) requires accurate identification and quantification of their presence before and after wastewater treatment.

Classical incubation and staining methods have been widely used to quantify hookworm ova from wastewater (Bastos et al., 2013; de Victorica and Galvan, 2003; Konate et al., 2013; Sharafi et al., 2012; US EPA, 2003), however, these methods have limitations. For instance, the incubation method requires up to 4 weeks (depending on the incubation temperature) to obtain results which may not be ideal for a situation where rapid health risk assessment is required (Boehm et al., 2009; Gyawali et al., 2015a). Similarly, the stain based methods require skilled personnel to differentiate hookworm ova from ova of other parasites such as *Oesophagostomum bifurcum* and *Trichostrongylus* spp. (Cabaret et al., 2002; Traub et al., 2007; Verweij et al., 2007). In addition, the performance of both these methods depends on the sensitivity of a microscope, which has been reported to be low. Weber et al. (1991) reported that the detection threshold of a microscope could be as low as 5%. This means that, 95% hookworm ova in a slide may not be detected during microscopic observation. In addition, hookworm ova on a slide must be observed within half an hour otherwise ova may escape detection (Verweij et al., 2007). Therefore, it is vital to develop a rapid, specific and sensitive method for simultaneous detection and quantification of hookworm ova from wastewater matrices.

We have recently developed a real-time PCR method for rapid, specific and sensitive detection of *Ancylostoma caninum* (dog hookworm) ova from wastewater matrices by targeting rRNA of Internal Transcribed Spacer (ITS-1) region (Gyawali et al., 2015a). *A. caninum* ova are genetically and morphologically very similar to human hookworm, and also occur in high numbers in dog faeces in Australia. Since it was difficult to obtain human hookworm in Australia, instead, *A. caninum* was selected as a surrogate for human hookworm. The detection sensitivity of the method was <1 ova/L of treated wastewater, < 4 ova per/L of raw wastewater and <4 ova/4 g sludge. The results can be obtained within 4–6 hr compared to incubation method that requires a longer time (up to few weeks). However, the real-time PCR method, however, cannot quantify amplified gene copy of target hookworm species.

While quantitative PCR (qPCR) can be used to quantify the numbers of pathogens in an environmental sample (Ahmed et al., 2014, 2015; Shanks et al., 2008), no information is available on the application of qPCR-based methods to quantify hookworm ova. Only a handful of studies had attempted to quantify *Ascaris* ova using qPCR under laboratory conditions (Pecson et al., 2006; Raynal et al., 2012). Pecson et al. (2006) created profiles of the ITS-1 rDNA and rRNA levels during the development of *Ascaris* eggs from single cells to third-stage larvae. The results of this study suggested that the accurate quantification of *Ascaris* ova can be challenging due to the presence of varying numbers of gene copies in *Ascaris* ovum depending on the development stage. It is highly likely that wastewater matrices may contain mixed population (early to late cell staged) of hookworm ova. Therefore, determining average gene copy numbers from a mixed population is essential to estimate the likely gene copy numbers per ovum. Such information may lead to the development of a qPCR assay to quantify hookworm ova from wastewater matrices. In view of this, an attempt was undertaken to estimate the numbers of hookworm ova from seeded wastewater and un-seeded wastewater matrices. The quantitative method developed in this study would aid in the risk assessment of hookworm associated with wastewater reuse.

1. Materials and methods

1.1. Source of hookworm ova for the study

Fresh dog faecal samples were collected from the School of Veterinary Science, University of Queensland, Australia. *A. caninum* ova were isolated within 48 hr after collection.

1.2. Development of qPCR assay

A 101 base pair (bp) of the 5.8S rRNA gene from ITS-1 region of *A. caninum* plasmid DNA sequence (TTTGCTAACGTGCACTGA ATGACAGCAAACTCGTTGTTGCTGCTGAATCGTTTACCGACTA TAAAACGTTTTGGCAGTGGCTAGTATGACAACGGTGTTTC) was purchased from Integrated DNA Technologies (IDT) (IDT Technology, USA). The 100 μL UltraPure™ water was added into the tube to obtain 40 ng/μL of plasmid DNA. Gene copy numbers were calculated by multiplying the DNA concentration by Avogadro's number and dividing by the product of the plasmid size (bp) and an average weight of a base pair (Yun et al., 2006). Serial dilutions were prepared ranging from 10^5 to 10^0 gene copies per μL and served as a standard. Previously published primers, F: 5′-TTT GCT AAC GTG CAC TGA ATG-3′ and R: 5′-GAA ACA CCG TTG TCA TAC TAG CC-3′, probes, P: FAM-5′-AAC TCG TTG TTG CTG CTG AA-3′-BHQ3 and cycling parameters, 95°C for 15 min, 40 cycles of 95°C for 15 sec and 59°C for 1 min were used to amplify the target gene (Gyawali et al., 2015a). The qPCR amplification was performed in 25 μL reaction mixtures containing 12.5 μL iQ™ Supermix (Bio-Rad Laboratories, USA), 250 nmol/L of each primer, 400 nmol/L of probe, 3 μL of template DNA and UltraPure™ DNase/RNase-free distilled water (Life Technologies, Australia). The qPCR assays were performed using the Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, USA).

1.3. qPCR reproducibility and lower limit of quantification (LLOQ)

The reproducibility of the qPCR assay was assessed by determining the intra-assay repeatability and inter-assay reproducibility. The coefficient of variation (CV) of the assay was calculated by analysing the standards. The intra-assay

Please cite this article as: Gyawali, P., et al., Quantification of hookworm ova from wastewater matrices using quantitative PCR, J. Environ. Sci. (2017), http://dx.doi.org/10.1016/j.jes.2016.10.019