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Towards enhanced automated elution systems for waterborne protozoa using megasonic energy

B. Horton[a,b,c], F. Katzer[a], M.P.Y. Desmulliez[d], H.L. Bridle[b,*]

[a] Moredun Research Institute, Pentlands Science Park/Bush Loan, Penicuik, EH26 0PZ, UK
[b] Institute of Biological Chemistry, Biophysics and Bioengineering, School of Engineering & Physical Sciences, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS, UK
[c] Moredun Scientifc, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, UK
[d] Institute of Sensors, Signals and Systems, School of Engineering & Physical Sciences, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS, UK

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ABSTRACT

Continuous and reliable monitoring of water sources for human consumption is imperative for public health. For protozoa, which cannot be multiplied efficiently in laboratory settings, concentration and recovery steps are key to a successful detection procedure. Recently, the use of megasonic energy was demonstrated to recover *Cryptosporidium* from commonly used water industry filtration procedures, forming thereby a basis for a simplified and cost effective method of elution of pathogens. In this article, we report the benefits of incorporating megasonic sonication into the current methodologies of *Giardia duodenalis* elution from an internationally approved filtration and elution system used within the water industry, the Filta-Max®. Megasonic energy assisted elution has many benefits over current methods since a smaller final volume of eluent allows removal of time-consuming centrifugation steps and reduces manual involvement resulting in a potentially more consistent and more cost-effective method. We also show that megasonic sonication of *G. duodenalis* cysts provides the option of a less damaging elution method compared to the standard Filta-Max® operation, although the elution from filter matrices is not currently fully optimised. A notable decrease in recovery of damaged cysts was observed in megasonic processed samples, potentially increasing the abilities of further genetic identification options upon isolation of the parasite from a filter sample. This work paves the way for the development of a fully automated and more cost-effective elution method of *Giardia* from water samples.

1. Introduction

Giardia duodenalis is a common waterborne protozoan parasite causing the diarrhoeal disease called 'Giardiasis' (Julio et al., 2012). This pathogen is thought to infect upwards of 200 million people per year, presenting itself therefore as both an economic and a public health burden throughout the world (Lane and Lloyd, 2002). The transmission route of the parasite is faecal/oral and parasite cysts are found within surface waters throughout the world. Although the disease is much more prevalent in the developing world, outbreaks of disease do still occur in the developed world, albeit at a lower level (Barwick et al., 2000; Feng and Xiao, 2011;). A study of waterborne disease outbreaks between 2004 and 2010 reported that *G. duodenalis* accounted for 70 out of the 199 reported outbreaks of human disease due to waterborne protozoa within the developed world (Baldursson and Karanis, 2011). In addition, food can easily become contaminated when washed with water containing infective cysts, emphasizing implications for food hygiene worldwide (Porter et al., 1990; Smith et al., 2007).

Cysts are well recognized as being resistant to the environment as well as to commonly used water treatment methods (Robertson and Lim, 2011b). Quantities of cysts required to cause infection vary depending on the host but the infectious dose in humans is typically between 10 and 100 cysts, subject to the immune status of individual hosts. Data generated by Robertson and Lim (2011a) suggest cattle and humans infected with *G. duodenalis* can shed up to 10^5 and 10^6 cysts/g of faeces, respectively. The quantities of immediately infectious cysts shed by hosts, combined with the impressive cyst longevity ranging from weeks to months highlight concerns for public health and widespread environmental contamination.

Currently there are regulatory requirements for water utilities within the UK for a similar protozoan species, *Cryptosporidium*, which has been extensively monitored in water since their introduction under the Water Supply (Water Quality) Regulations, 1999, SI No. 1524. The UK regulations, enforcing this regulation, have had a positive impact on reducing outbreaks of *Cryptosporidium* in the public. Data presented by Robertson and Lim (2011c), based on the work by Lake et al. (2007),

* Corresponding author.
E-mail address: h.l.bridle@hw.ac.uk (H.L. Bridle).

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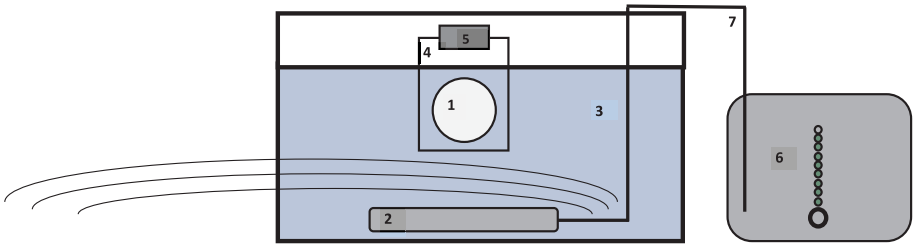

Fig. 1. Schematic setup of the megasonic bath during membrane sonication. Samples can be seen submerged in the water bath and exposed to megasonic waves generated by the megasonic transducer. Samples were sonicated for 20 min in this fashion. 1) Filta-Max® membrane or sponges within the Filta-Max® module, 2) Megasonic transducer generating megasonic waves, 3) Water filled bath, 4) Partially submerged plastic bag containing Phosphate Buffered Saline with Tween®-20 (PBST) and membrane and 5) adhesive tape sealing the plastic bag 6) Megasonic control and power supply 7) Water protected cabling.

suggest that, in North England, 905 cases representing around 7000 potential infections were prevented since its implementation to 2007. A Standard Method (ISO15553:, 2006) is also utilised, within Europe and internationally, for *Cryptosporidium* and *Giardia* detection, though not all countries implement regulatory monitoring of these pathogens. Although not monitored within the UK, *Giardia duodenalis* has become extensively monitored through the US EPA Method 1623 (Method 1623.1, 2012) in the US, following the EPA Safe Drinking Water Act (SDWA) in 1997. The enforcement of this method has been very effective in reducing outbreaks within the USA, the country with the most reported outbreaks of Giardiasis in the world (Robertson and Lim, 2011d). Delay between point of infection and clinical disease, and varying clinical symptoms displayed in individual infections, have however led to potential large-scale under-reporting of the parasite throughout the UK, and indeed the world.

To implement the US EPA Method, the ISO15553 Standard Method and the UK Water Quality Regulations, filtration of *Cryptosporidium* and *Giardia duodenalis* from water samples most commonly utilises the Filta-Max® system though EnviroChek® capsules are also applied, which allow simultaneous recovery of both pathogens. Current filtration recoveries for *Giardia duodenalis* cysts in reagent grade water vary between 41% to 70% according to the UK Environment Agency (2010), as confirmed by many authors over the years, with mean recoveries of 49.8 ± 5.4% (Wohlsen et al., 2004) or 56.7 ± 22.2%, (UK Environment Agency, 2010) for example.

Megasonic sonication operates in a similar way to that of ultrasonic sonication, albeit at a higher operating excitation frequency. Typically megasonic sonication utilises a frequency of over 1 MHz created by a piezoelectric transducer, which is placed within a fluid filled container. When powered, the transducer creates high frequency sound waves, which move through the fluid and oscillate through maximum and minimum pressure loci along the wave. Bubbles are created at the points where the minimum pressure along the wave lie below the vapour pressure of the liquid; these bubbles then collapse upon exposure to the maximum pressure of the wave. This implosion of bubbles, called cavitation, creates a much kinder elution process compared to that of ultrasonic sonication, due to the bubbles being smaller in size, thus liberating less cavitation energy and creating less local turbulence at the time of collapse (Kerrouche et al., 2015; Jones et al., 2016; Chitra et al., 2004). Ultrasonic sonication methods are already employed in many applications requiring harsh treatment, which include cleaning equipment, disrupting cells or killing pathogens. Megasonic sonication has shown promising results in the elution of *Cryptosporidium parvum* from Filta-Max® matrixes, as reported by Kerrouche et al. (2015). Without the use of surfactants, the method has demonstrated recovery rates comparable to the commonly used methodology implemented in the UK internationally within the water testing community (The UK Environment Agency, 2010). The megasonic technique also displayed a range of additional economic and financial benefits over the current method, such as savings in time and manpower resource through the removal of the centrifugation process and non-automatable steps.

In an attempt to improve the current methods of *G. duodenalis* filtration using the Filta-Max® system, we incorporate here megasonic sonication in the elution stage of the standard protocol of *Giardia duodenalis*, and investigate whether the same benefits can be reported as with *Cryptosporidium*. In addition, we examine the impact of the megasonic sonication method with regard to the integrity of *G. duodenalis* cysts during elution.

2. Materials and methods

The aim of this study was to assess the efficacy of megasonic elution from a Filta-Max® system in increasing the recovery rate of *Giardia duodenalis* either from the membrane filter or from the sponges composing the Filta-Max® sponge filter module. Based on previous work carried out on *Cryptosporidium parvum* by Kerrouche et al. (2015), three experiments were carried out to individually study steps in the protocol: (1) seeding *Giardia duodenalis* into sponges within a Filta-Max® sponge filter module to investigate the impact of recovery rate in this first stage; (2) seeding *Giardia duodenalis* into Phosphate Buffered Saline with Tween®-20 (PBST) eluent prior to concentration through a Filta-Max® membrane filter; and finally (3) direct seeding of *Giardia duodenalis* cysts onto the Filta-Max® membrane filter in order to determine the impact on recovery rates of using megasonic elution with the membrane filter in two different operating modes. The stages are indicated in Fig. 1 by a bracket and numbering. Finally, an overall procedure for megasonic elution, combining 1 and 3 above, was compared to the standard protocol.

A schematic of the experimental setup is presented in Fig. 2. The megasonic sonicator plate, labelled as 2 in Fig. 2 and manufactured by the Company Sonosys (Sonosys, 2016), is a square-faced submersible device composed of four rectangular, 2.5 cm × 11 cm piezo-transducers embedded in a steel sheet of 1.03 dm^2 area. The piezoelectric transducers emit an acoustic wave of frequency of 1 MHz ± 0.05 MHz with a power adjustable by the megasonic transducer control panel. At maximum power, 500 W of electrical power is supplied to the piezoelectric emitter with conversion efficiency to acoustic power output of around 90%. The samples to be eluted, labelled 1 in Fig. 2, are facing the transducers (different orientation in the figure) so that the megasonic wave is fully incident onto the samples surface.

In all Filta-Max® experiments samples were spiked with EasySeed suspensions containing 100 Gamma-irradiated *Giardia duodenalis* cysts (TCS Biosciences, UK). Non-Gamma irradiated cysts (Waterborne, Inc. USA) were only used for a viability study. Gamma irradiated cysts were utilised for all the Filta-Max® experiments due to the counted reliability in the numbers of cysts inside the suspensions (100 cysts ± 2). A difference between gamma-irradiated and live *Giardia duodenalis* cyst shell dynamics could potentially affect recoveries when compared to other studies using live cysts. On consulting TCS Biosciences, the distributor of the EasySeed cysts in the UK, knowledge of how well the irradiated cysts represent live parasites during filtration is unknown. For this reason both field and laboratory trials using live cysts and megasonic elution would be useful. Previous work has highlighted that the performance of the GC-Combo Dynabead kit for Immunomagnetic