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MagnaExtract, a novel magnetic bead-based extraction method for the molecular detection of antimicrobial resistance genes in fresh water.

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ABSTRACT

Background

Environmental water samples are increasingly recognised as an important reservoir of antimicrobial resistance (AMR) genes. Polymerase chain reaction (PCR) and next generation sequencing (NGS) offer a potentially inclusive surveillance platform for a wide range of AMR genes. However, molecular methods are dependent upon the extraction of DNA of high yield and quality. Current options for DNA extraction from complex environmental matrices for downstream molecular applications are either expensive or low yielding. We present here a novel magnetic bead-based DNA extraction method, for the detection of antimicrobial resistance genes (ARGs) from river water in Malawi, named MagnaExtract.

Methods

MagnaExtract involves initial filtration of 250ml freshwater, followed by an overnight incubation of the filter in 15ml buffered peptone water (BPW), common procedure in microbiology laboratories. 200µl is then taken for a boil (95°C) and spin step and mixed with magnetic beads to bind DNA. Following washes with ethanol, the DNA is eluted in nuclease-free water. To determine the effectiveness of this method, 98 freshwater samples were collected from two rivers in Southern Malawi, and DNA was isolated using the MagnaExtract method, two commercial Qiagen (Germany) kits; PowerWater and DNeasy Blood and tissue, alongside a boil and spin of BPW, and a boil and spin from bacterial isolate grown on agar media. All samples were screened with a high-resolution melt (HRM) PCR panel previously validated for the detection of third generation cephalosporin and carbapenem ARGs. We compared the DNA yield obtained using all extraction methods, as well as the identification of each ARG.

Results

DNA yield using MagnaExtract was statistically greater than both boil and spin methods and DNeasy Blood & Tissue (Qiagen, Germany). DNA yield was slightly lower than using PowerWater (Qiagen) but the difference was not statistically significant. MagnaExtract was the only method to identify

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ARGs in all 98 water samples compared with PowerWater (n=82), DNeasy (n=95) boilate of BPW (n=75) and boilate of bacterial isolate (n=87). The most commonly detected ARG was OXA-48 (n=93). In addition, we found overnight incubation in non-selective enrichment broth (BPW) to promote the growth of bacteria harbouring extended spectrum beta lactamase (ESBL) genes and reduction in the detection of carbapenemase genes.

Conclusion

The MagnaExtract approach offers a simple, affordable, high yielding DNA extraction method for the detection of ARGs isolated from river water samples.

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BACKGROUND

A key component of the response to the global public health threat of antimicrobial resistance (AMR) is improved surveillance, however barriers still exist to accurate and reliable diagnostic and surveillance tools. This is highlighted in the World Health Organisation (WHO) global action plan on AMR, which articulates the need for improved diagnostic and surveillance assays in three of the plan's five strategic objectives (WHO, 2017). One technical approach to AMR surveillance is the usage of molecular diagnostics. For example, nucleic acid amplification approaches such as polymerase chain reaction (PCR) and next generation sequencing (NGS) can be used to investigate and describe the genotypic profile of bacteria and thus infer their AMR status (Argimón et al., 2020). PCR and WGS are both critically dependant on the isolation of high-quality DNA (Gupta, 2019; Mantere et al., 2019).

The quality of DNA extracted depends on two principal factors: the original sample type and the extraction methods used (Dhaliwal, 2013; Surzycki, 2000). For pure samples such as cultured bacterial cells, with sufficient starting material, DNA yield is typically high (Gabor et al., 2003). More complex samples, such as environmental water sources, may contain diverse inhibitors such as salts, DNases and humic compounds that lead to a vastly reduced DNA yield, and inhibition of molecular analysis including PCR and NGS (Williams et al., 2017).

The environment, especially water sources, is increasingly recognised as an important source of antimicrobial resistant genes (ARG) (Devarajan et al., 2016; Sanderson et al., 2018; Stoll et al., 2012), with recent studies demonstrating their widespread prevalence in surface water samples (Ng & Gin, 2019; Waseem et al., 2017). A critical question in the epidemiology of AMR is the degree to which there is flux between human, animal and environmental compartments resulting in a One Health approach being adapted in many settings. A large proportion of these are set in high-income countries and only report on culturable bacteria in river water (Harwood et al., 2000; Henriot et al., 2019; Servais & Passerat, 2009; Stoll et al., 2012). Only a small proportion (<0.1%) of aquatic microbes are culturable on agar media by standard methods, which can bias results (Amann et al., 1995; Stoll et al., 2012). Molecular diagnostics, in particular metagenomic approaches, have the potential to offer an

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"inclusive" platform to survey the entire diversity of ARGs present in a given sample, rather than just the culturable subset, as long as adequate DNA can be extracted from these complex matrices.

Once environmental water has been collected, the next key question is how to process such samples without a loss of diversity prior to DNA extraction. There is wide acceptance that water samples should be concentrated by filtration prior to extraction, in order to maximise organism capture (Deiner et al., 2015; Eichmiller et al., 2016; Hinlo et al., 2017; Piggott, 2016). There is less consensus about the use of an overnight incubation step in enrichment broth within certain settings despite its regular use in microbiological procedures (da Silva et al., 2012). In the context of AMR surveillance there may be pros and cons to this approach; whilst target organisms (i.e. *Escherichia coli*) may be amplified, important ARGs on mobile genetic elements (i.e. plasmids) may be lost during this step due to a suspected fitness cost (Huang et al., 2013). Equally, human adapted or human restricted pathogens i.e. *Salmonella* Typhi may be outcompeted in such media.

Commercially available kits are typically used for DNA extraction from environmental samples, as they offer standardised sets of reagents and are safer than phenol-chloroform-isoamyl alcohol (PCI) extraction methods (Hinlo et al., 2017). Adaptations of manufacturer's instructions are often reported (Barta et al., 2017; Renshaw et al., 2015), but novel methods are rarely incorporated in high-throughput studies (Oberacker et al., 2019). This has led to inconsistent application of such kits amongst environmental researchers and often the kit used is determined by cost, accessibility of materials or personal preference (Hinlo et al., 2017). Rudimentary DNA extraction methods have been developed such as the boilate technique (Dashti et al., 2009). Boilate requires only a heat block for cell lysis and a microcentrifuge to pellet the DNA and remove cellular debris, apparatus present in most laboratories around the world, however the lack of a concentration step often results in a lower yield in comparison with commercial kits (Williams et al., 2017).

In recent years, there has been increased interest in the use of magnetic particles for DNA and protein purification (Ma et al., 2013; Naresh et al., 2011; Oberacker et al., 2019). Magnetic beads can be coated with a DNA loading antibody or a functional group that specifically interacts with DNA. After binding the DNA, beads are separated from other contaminating cellular components and then

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purified by ethanol washing. Their utility has, to date, been limited by the high cost of commercially available beads and the lack of open-source methodologies for laboratory developed beads (Oberacker et al., 2019). In addition, most available protocols require chemical reagents for lysis and precipitation that can be inaccessible in resource-limited settings.

Here we present an affordable and novel magnetic bead-based extraction method for the isolation of bacterial DNA using Malawian river water samples as a model. This is directly compared to two commercially available QIAGEN kits and the rudimentary boilate method using the same samples by testing the DNA yielded from each method with an in-house multiplexed HRM-PCR assay.

METHODS

Setting

As part of an ongoing AMR surveillance project, Drivers of Resistance in Uganda and Malawi (DRUM) households were randomly selected based on their geographical location within regions of Southern Malawi (Drum, 2020). Household members were asked to identify their source of river water and field sampling sites were subsequently selected based on their ease of access. Ethical approval for this study was obtained from the University of Malawi College of Medicine Research Ethics Committee (COMREC: P.11/18/2541) and Liverpool School of Tropical Medicine research and ethics committee (LSTM REC: 18-090)

Sample collection and processing

Ninety-eight water samples were collected in sterile 250ml plastic containers and issued a unique identification number. Samples were stored in ice chests, transported within two hours of collection to our laboratory for reception and stored at 4°C for a maximum of 24 hours prior to processing. All samples were then concentrated using a pump water filtration system of optimum flow rate 3.8-4.0 L/min and passed through VWS Supor® PES membrane filters of aperture 0.45µm (PALL, USA). The filter paper was then cut in two: half was available for immediate DNA extraction and the other incubated overnight in 15ml of buffered peptone water (BPW; Oxoid Limited, UK) at 37°C.

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Comparator DNA extraction methods

DNA was directly extracted from the filter paper using the PowerWater kit (QIAGEN, Germany) following the manufacturer's instructions. After overnight incubation, 200µl of BPW was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Germany), 200µl by the boilate method; heated at 95°C for 10 minutes and centrifuged at 8000RPM for 5 minutes with the supernatant retained for downstream application. A further 200µl was extracted using the novel MagnaExtract method. Finally, 10µl was used to inoculate a Chromagar™ extended spectrum beta-lactamase (ESBL) ESBL plate (CHROMagar, France), that phenotypically infers ESBL resistance and incubated overnight at 37°C. A plate sweep was performed by an experienced microbiologist (MM) to include all morphologically distinct colonies present on the plate. This was then suspended in 200µl of distilled water and DNA isolated using the boilate method.

Thus, for all samples, five different DNA samples were obtained, as shown in Figure 1. The DNA concentration of each sample was quantified using the ThermoFisher Qubit fluorometer 2.0 broad range double stranded DNA kit and stored at -20°C to await PCR analysis.

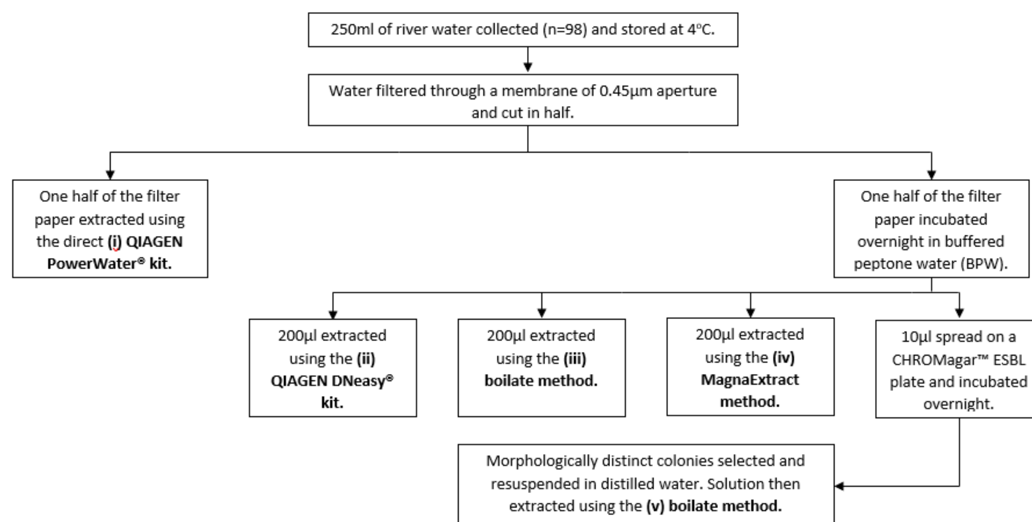

***Figure 1**: A schematic overview of the methods of DNA extraction utilised*

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The MagnaExtract method

The MagnaExtract protocol was optimised prior to sample collection, with the addition/absence of proteinase K, incubation duration, bead, ethanol and elution volume optimised using water spiked with UK laboratory isolates of *Escherichia coli* (EC172) and *Klebsiella pneumoniae* (KLD50395) . The final protocol presented here recorded the maximum DNA yield and earliest qPCR amplification and was applied for sample testing.

MagnaExtract utilises the boilate method with a starting material of 200µl of BPW, heats for 10 minutes at 95oC and then centrifuges at 8000RPM for 5 minutes. MagnaExtract continues by mixing 100µl of supernatant with 100µl of diluted prepared magnetic beads (see supplementary material for preparation methodology) (Fouet et al., 2017; Rohland & Reich, 2012),and incubating at room temperature for 5 minutes. The solution was then pelleted on a magnetic rack and the supernatant discarded. The beads were washed with 500µl of 70% ethanol, pelleted again on the magnetic rack and the supernatant discarded; 200µl of 70% ethanol was used for a final wash step and the beads were concentrated on the rack. The DNA was then eluted in 30µl of molecular grade water, and carefully removed from the pelleted magnetic beads.

High resolution melt (HRM) analysis for the presence of antimicrobial resistance genes (ARGs)

Primers for ESBL ($bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-9}}$ group and bla_{SHV}) and carbapenemase (bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$, and bla_{VIM}) genes were taken from a previously published assay ((Edwards et al., 2019)). Each reaction of the HRM AMR gene assay included 6.25µl of Type-IT 2 x HRM buffer (Qiagen, Germany), the primer mix as previously defined, and molecular grade water was added to make a final volume of 12.5µl, including 2.5µl of sample DNA. Reactions were thermally cycled in a RGQ 6000 (Qiagen) model thermocycler, using the profile described by Edwards *et al.,* 2019. The threshold value for cycle threshold (Ct) was set at 0.078 dF/dT and retained for all experiments. All analysis was performed in the RGQ software. The presence of carbapenem resistance genes,

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previously unreported in Malawi were confirmed by in-house single-plex qPCR assays using hydrolysis probes.

Quantitative and cost analysis

Data handling, analysis and statistical comparisons were all performed using R (3.5.5) (R, 2020) Statistical analyses for DNA yield were performed using Kruskal-Wallis one way analysis of variance with Dunn's post-hoc test to identify differences in yields using each of the five extraction methods. Overall agreement was determined using a composite reference standard inclusive of all extraction methods utilising PCR results.

$$Agreement = \frac{\text{Number of positives by the extraction method of interest}}{Total\ number\ of\ positives\ inclusive\ of\ all\ five\ extraction\ methods}$$

Cost was estimated for all extraction methods inclusive of laboratory consumables from sample collection to DNA elute for each process.

RESULTS

In total, 98 samples were collected and extracted for each of the five methods outlined in Figure 1. Of these, 19 of the samples did not have sufficient extractant volume remaining to conduct DNA yield quantification in one or more of the extraction methods. Therefore, 79 samples were tested for DNA yield.

DNA Yield

DNA yield varied significantly between the five different extraction methods. The overall DNA yield using MagnaExtract was statistically greater than that achieved using DNeasy, boilate of BPW or boilate of cultured isolate ($p < 0.0001$), Kruskal-Wallis test, Dunn's post-hoc test, (n=79) (Figure 2). The DNA yield using the PowerWater kit per manufacturer's instructions was not significantly different to that of MagnaExtract ($p = 0.82$), however there was greater variance between samples when compared (Table 1). Of note, no DNA was recovered from 5/79 samples using PowerWater, whereas MagnaExtract was able to detect DNA in all samples.

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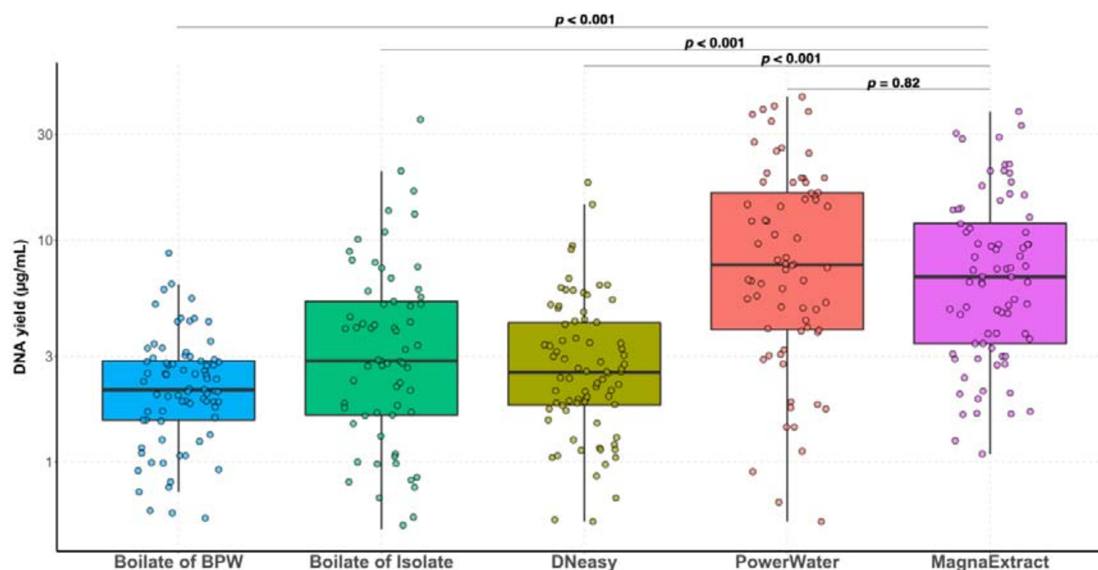

Figure 2: **The yield of DNA (µg/mL) from Malawian river water samples (n=79) utilising five different extraction methods**. The MagnaExtract method is shown to have statistically higher DNA yield than DNeasy, boilate of isolate, boilate and BPW ($p < 0.0001$) and reported comparable values to PowerWater ($p = 0.82$).

Table 1: Comparison of DNA extraction methods for the recovery of bacterial DNA in Malawi river water. Cost of DNA extraction methods calculated per sample including cost of commercial kits and all laboratory consumables. Time of extraction method determined from start of extraction to DNA elution. The percentage of positive samples that were correctly identified as positive (agreement), utilising a composite reference standard. Median DNA yield and 5-95 confidence interval for all DNA extraction methods for each extraction method.

Extraction method	Cost (£)	Time (hr)	Agreement (%)	Mean DNA yield (µg/ml) [5-95 CI interval]
MagnaExtract	1.43	0.5 [a]	**100**	6.87 [1.66 - 30.56]
PowerWater	8.38	1-1.5	82	**7.70 [0.01 - 44.97]**
DNeasy blood and tissue	5.38	1-1.5[a]	75	2.52 [0.85 - 6.96]
Boilate of BPW	**0.66**	**0.25** [a]	95	2.09 [0.6 - 5.18]
Boilate of isolate	2.04	**0.25** [b]	87	2.69 [0.01 - 13.22]

For time of extraction method from river water collected to DNA elute, [a]24 hours and [b] 48 hours should be added.

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Cost and time

The commercial kits demanded the greatest cost and laboratory time whilst reporting the lowest positive agreement between samples, although PowerWater did not require overnight incubation which will be beneficial in some settings, see table 1. Boilate of BPW was the cheapest and quickest method but yielded the least DNA. MagnaExtract offered an inexpensive yet high yielding DNA extraction method, with the highest positive agreement.

Molecular detection of ARGs

Of the 98 river water samples collected, 98.9% (n=97) were positive by PCR by one or more extraction method for one or more ARGs and organisms were isolated from 92.8% (n=91) on ESBL-selective agar by plate culture. Only the MagnaExtract method was able to identify ARGs in all composite molecular positive samples compared to the reference standard (table 1) with the lowest agreement reported by the DNeasy blood and tissue kit. There was little agreement between each method in terms of which ARG were detected within each individual sample (Figure 3). $bla_{\text{OXA-48}}$ (n=93) was the most prevalent ARG followed by the ESBL ARGs ($bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-9}}$ group and bla_{SHV}). The number of ESBL ARGs detected was greater for methods utilising an overnight enrichment step than PowerWater extracted directly on a filter paper (Figure 3a, b c and e). Whereas the majority of carbapenemase ARGs (bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$, and bla_{VIM}) were identified by the direct PowerWater kit extraction, no carbapenemase ARGs were detected in colonies isolated on ESBL selective media and extracted using the boilate method.

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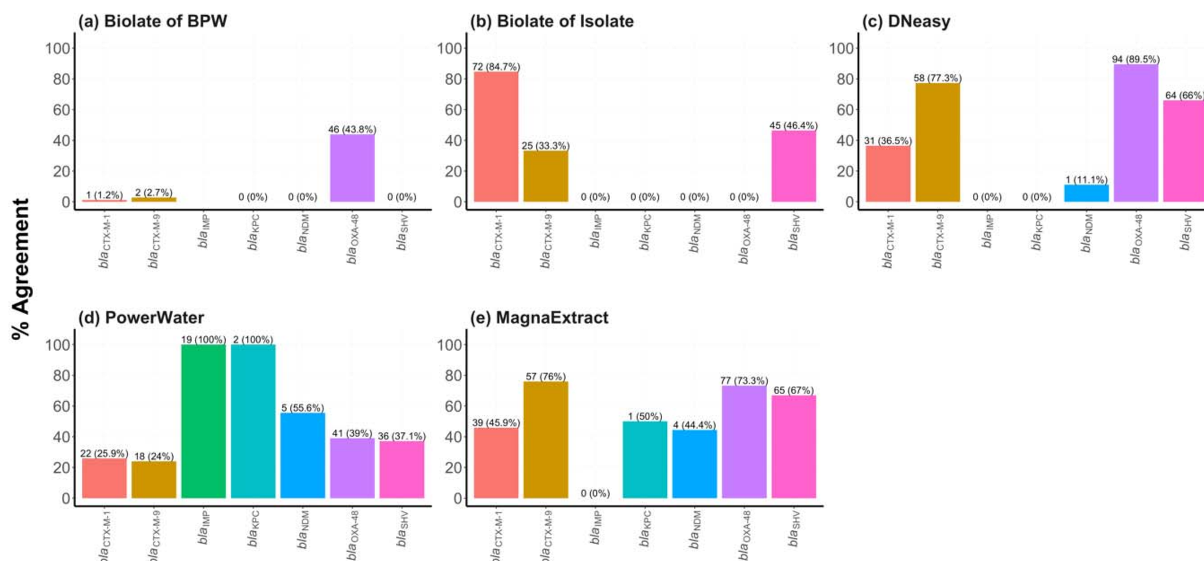

Figure 3 AMR genes detected using five different methods (**(a)** Biolate of BPW, **(b)** Biolate of Isolate, **(c)** DNeasy, **(d)** Power Water and **(e)** MagnaExtract), the y-axis represents the % Agreement for each method where each bar is the percentage of total unique hits using one method compared to total unique hits using all methods combined. Numbers above each bar represent the total number of unique hits for each gene using the corresponding method and in brackets is the % Agreement.

There was greatest agreement of ARG detection between MagnaExtract and the two commercial kits utilised in this study: PowerWater used on the direct filter samples and DNeasy performed at the same time point as MagnaExtract. However, consistent reporting of ARGs was poor as shown in figure 4. Despite undergoing the same pre-extraction steps, MagnaExtract was able to identify a higher proportion of clinically important ARGs ($bla_{CTX-M-1}$ group, $bla_{CTX-M-9}$ group, bla_{SHV} and bla_{NDM}) quicker and at a lower cost than DNeasy (see table 1). Detection of ESBL ARGs was notably lower direct from filter samples than DNeasy and MagnaExtract, suggesting an advantage of overnight incubation for ESBL detection. Tangentially, carbapenemase ARGs, with the exception of bla_{OXA-48}, were consistently underreported after the same incubation step.

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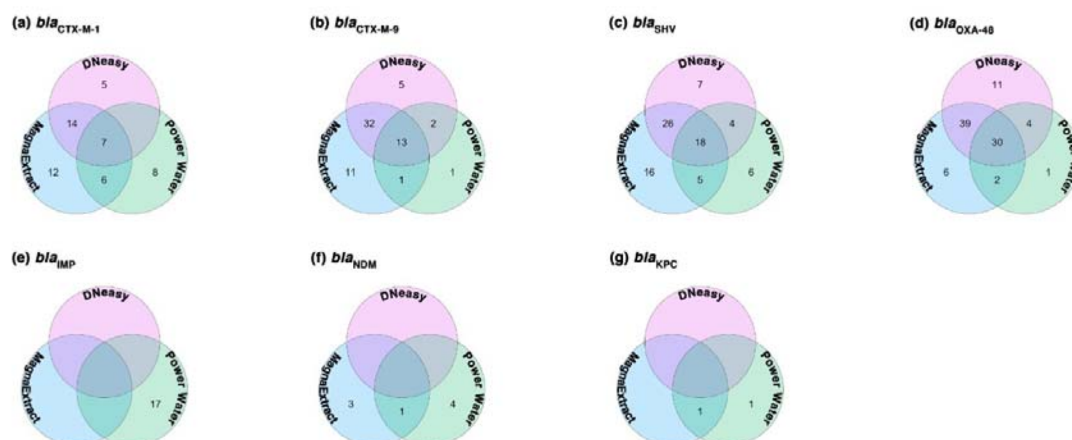

Figure 4: Venn diagrams showing the intersections for the detection of antimicrobial resistance genes (ARGs) extracted from 98 Malawian river water samples using three different methods. *Each diagram represents one ARG belonging to either the ESBL class of resistance markers (*$bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-9}}$ group, bla_{SHV}*) or carbapenemases (*bla_{IMP}, bla_{KPC}, bla_{NDM} and $bla_{\text{OXA-48}}$*). Each section of the venn is a different extraction method used (commercially available kits PowerWater and DNeasy (Qiagen, UK) and our novel MagnaExtract magnetic bead-based method).*

DISCUSSION

The MagnaExtract protocol is high-yielding and cost-efficient

Commercially available kits remain the extraction method of choice despite high consumable cost, lengthy laboratory time and varying DNA yield. Rudimentary methods offer quick, robust and cheap alternatives but can be unreliable and inhibited by humic substances leading to a lack of uptake. Here we report an inexpensive and high yielding DNA extraction method for the detection of ARGs from river water, employing diluted magnetic beads termed 'MagnaExtract'. This method concentrates bacteria by filtration followed by overnight incubation in a non-selective growth media (BPW) as is standard practice in many environmental microbiology laboratories globally (Djurhuus et al., 2017). By using solid-phase DNA capture with ethanol washes to remove inhibitors, MagnaExtract can incorporate a concentration step absent from most rudimentary methods without the need for expensive chemicals such as the wash buffers and spin columns used in commercial kits. This reduction of cost may be beneficial in low-middle income countries (LMICs), where the threat of AMR is greatest and improved surveillance is needed (Ayukekbong et al., 2017).

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Our novel MagnaExtract method yielded significantly higher amounts of DNA than both commercial (DNeasy, QIAGEN) and rudimentary methods (Boilate of BPW and isolates from cell culture), and is statistically similar to PowerWater (Qiagen, Germany). Whilst the PowerWater method is quicker than MagnaExtract as it does not require overnight incubation, environmental sampling is not as time-critical as clinical sampling where time to result may be of the upmost importance.

ARGs in Malawian river water

Concurrently, we present the first report of the following carbapenemase resistance genes $bla_{\text{OXA-48}}$ (n=94), bla_{IMP} (n=13), bla_{NDM} (n=8) and bla_{KPC} (n=3) in Malawian river water, although bla_{NDM} have previously been reported clinically (Lewis et al., 2020). Whilst the ARGs detected cannot be attributed to a specific bacterial species, water sources are susceptible to anthropogenic pressures and are often polluted with antibiotics and both commensal and pathogenic bacteria associated with humans (Sanderson et al., 2018).

Antibiotics can enter the environment from human and animal waste, particularly in areas of intensive farming or aquaculture, and may exert selective pressure if in sufficient quantity (Huijbers et al., 2019; Von Wintersdorff et al., 2016). Plasmids containing ARGs often harbour genes providing resistance to surfactants, solvents and other biocides, which may also be found in river water, and again select for ARG persistence (Singer et al., 2016). Additionally, some ARGs in certain species have been shown to have no fitness cost and may be carried indefinitely (Di Luca et al., 2017). The persistence of these genes in the environment can potentially pose a transmission risk to humans via water usage for drinking or agriculture. Surveillance of river water enables the assessment of which ARGs are circulating in the environmental population, whilst offering cheap, non-invasive indicator of local resistance levels to specific antibiotics.

The effect of overnight incubation on the recovery of ARGs

The effect of overnight incubation on the recovery of ARGs has been explored in this study. This incubation step amplifies bacteria, and therefore target ARGs, in the sample, improving test sensitivity. This comes at the expense of altering the microbial composition of the original sample,

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depending on the growth characteristics of present organisms in the culture conditions. Here, we show the inability to detect multiple carbapenemase resistance genes (bla_{NDM}, bla_{IMP} and bla_{KPC}) after 24 hours in non-selective enrichment media, and in contrast report a greater number of ESBL resistance genes detected post incubation than following direct DNA extraction from filter paper. This is likely due to the differential growth rates between organisms within the initial sample, and the potential of plasmid loss during culture. There has been an increase in understanding of the relative fitness costs caused by carriage of ARG genes to bacteria, notably those associated with large mobile plasmids which can be lost when antibiotic selection pressure is removed (Huang et al., 2013; Melnyk et al., 2015). This study was not designed for an in-depth investigation of the effect of overnight incubation, but the loss of carbapenemases post incubation requires further investigation.

Conclusion

MagnaExtract in combination with multiplex qPCR offers a cheap and rapid method for the molecular detection of antimicrobial resistance genes from complex river water matrices. We have shown that it is a reliable alternative to more expensive commercially available kits which will benefit future environmental AMR surveillance strategies.

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Supplementary material: Preparation of magnetic beads for MagnaExtract method.

1. Mix sera-mag SpeedBeads and transfer 1ml to a 1.5ml centrifuge tube.
2. Place Speed Beads on magnetic rack until beads are separated.
3. Remove supernatant.
4. Add 1ml TE (pH 7.5-8.0) to beads, remove from magnet, mix by pipetting up and down return to magnet.
5. Remove supernatant.
6. Repeat steps 4 & 5.
7. Add 1ml TE (pH 7.5-8.0) to beads, remove from magnet, mix by pipetting up and down, but DO NOT return to magnet.
8. Add 9g PEG-8000 to a new 50ml conical tube.
9. Add 2.92g NaCl to conical.
10. Add 500ul 1M Tris-HCl to conical.
11. Add 100ul 0.5M EDTA to conical.
12. Fill conical to 49ml using ddH20.
13. Mix conical for 5 minutes until PEG goes into solution.
14. Add 25ul Tween 20 to conical and mix.
15. Add SpeedBead and TE solution from step 7 to conical and mix.
16. Fill conical to 50ml mark with ddH20.