DEVELOPMENT AND TESTING OF AN ISOTHERMAL AMPLIFICATION AND LATERAL FLOW ASSAY TO DETECT SELECTED PATHOGENS IN WATER

Report to the
WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

BACKGROUND

Sustainable living and One Health principles demand increased surveillance of our environment to ensure a healthy environment, animals, and humans. The use of water resources of variable quality for human and agriculture needs necessitates improved, more sensitive, rapid detection of potential viable and pathogenic microbes in water. While microbial culturing remains the gold standard for diagnostic monitoring of water, molecular detection of pathogens combined with proof of viability of detected microbes are gaining ground to do diagnostic surveillance, especially to detect microbes not routinely tested for. This project was aimed at developing a rapid detection method for selected bacterial pathogens using a combination of isothermal polymerase chain reaction (PCR) method and detection of PCR products using lateral flow.

AIMS

The following were the aims of the project:

1. To develop an isothermal PCR method and detect amplicons using a lateral flow device,
2. To test the optimised method using real world samples, namely various types of water (environmental, tap, treated sewage and reclaimed water).

METHODS

An isothermal DNA amplification method named recombinase polymerase amplification, specifically using a commercial preparation produced by a company named TwistDx was used to extract DNA and produce amplicons from cultured Escherichia coli 0157:H7, Clostridium perfringens and Legionella pneumophila. The amplicons produced were detected using commercially produced lateral devices. The initial methods used in this study were conventional PCR, Sanger sequencing, real-time PCR using SYBR-I chemistry. Finally, optimised conditions were employed with recombinase polymerase amplification and detection by LFA devices. To test, the optimised methods, environmental water samples were collected and filtered, and filters rinsed off and used for culturing and PCRs. As different water types represent different challenges for PCR, filtering and DNA extractions were optimised to inform on ideal methods fit for specific water types.

RESULTS AND DISCUSSION

1. Detection of DNA using a Recombinase Polymerase Amplification (RPA) PCR method

An isothermal PCR to detect the *Clostridium perfringens* phospholipase C gene was successfully developed. This includes designing primers for recombinase polymerase amplification, optimising the amount of primer, DNA template, the RPA PCR temperature and duration of the PCR. The *plc* target was successfully amplified using approximately 25 ng of template, and a PCR temperature of 37ºC was shown to be optimal and 30 minutes of amplification minimal to obtain clearly visible amplicons on agarose gels. For *E. coli* 0157:H7 and *L. pneumophila*, previously described RPA PCR methods were successfully carried out to detect DNA. In this study, 3 different kits, a magnetic bead extraction method, a column based method and a modified commercially available chemical clean-up method were tested for DNA extraction, and results obtained showed that all of them were suitable for downstream PCR amplification and detection. Extracted DNA, from all 3 kits, were used successfully in both conventional and isothermal PCR (specifically recombinase polymerase amplification). Using serial dilutions of pure DNA, we showed that input DNA as low as 10 picogram of genomic DNA could be detected using PCR and detection with both agarose gel electrophoreses and a lateral flow device. Amplicons generated from 1 picogram of DNA was barely visible and required manipulation of UV exposure to be visible on gels (thus not reliable with the conditions used for this study, but potentially PCR conditions could be optimised further).

2. Lateral flow detection of amplified DNA

The procedure described in the PCRD manual (Abingdon Health, UK), to generate dual labelled amplicons and detecting it with the PCRD lateral flow cassette, was followed: for each bacterium, the primer was labelled with biotin, while the forward primers was labelled with either 5-FAM (a fluorophore) or DIG. Using this in a

PCR generates amplicons labelled with biotin on one end and either 5-FAM or DIG on the other end. The labelled primers worked well both for conventional and isothermal PCR and was successfully detected with the PCRD lateral flow device.

In this study, both a liquid RPA reaction mix, as well as a lyophilised version from the same company were tested for use with the lateral flow device. Both the liquid and lyophilised kits worked well, however, there are some issues worth noting. First, the liquid version contained a buffer that is extremely viscous and therefore requires proper pipetting technique (experienced technician). A second concern is that the liquid kit comes in a big volume, so it would be best to do aliquots of the various reagents and freeze them. On the other hand, the lyophilised version is provided in individual PCR tubes and do not even need cold storage. However, dissolving the reagents also required patience and proper pipetting (easily made bubbles). The PCR volume is 50 µl and thus a waste of reagent, in this study an attempt was made to try dissolving and splitting one tube into 2 reactions, however, this was not successful. Thus, it is recommended that any laboratory using this specific commercial kit (currently only one available worldwide), use the lyophilised version of this RPA reagent.

Another concern was that RPA and other isothermal methods, generally generate more amplicons than conventional PCR, which could result in a messy lateral flow detection. However, detected bands were observed within five minutes of loading onto the lateral flow devices and only non-specific detection (on the other test line) if the cassette was read after 10 minutes. This suggest that there could be some optimisation needed but should be tested with real environmental water samples.
To conclude, the principle for detection of amplicons generated using an isothermal PCR amplification method, combined with a commercially available, readymade lateral flow device was successfully demonstrated.

SUMMARY OF FINDINGS

In summary, the following was achieved in this project:

- The successful design and testing of a new primer set for the development and optimisation of a recombinase polymerase amplification PCR to detect the phospholipase (*plc)* gene of *Clostridium perfringens*, validated by conventional PCR and Sanger sequencing of generated amplicons (PCR products).
- Previously described primers used for recombinase polymerase amplification of *Legionella pneumophila* and *E. coli* 0157:H7 were verified. For this, Sanger sequencing was used to confirm that the primers resulted in the amplification of the correct PCR products
- The primer sets for the 3 PCRs, to detect *Clostridium perfringens*, *L. pneumophila* and *E. coli* 0157:H7 were successfully tested using DNA extracted from pure, cultured reference isolates of these organisms. Extracted DNA was tested both with conventional PCR and recombinase polymerase amplification (RPA). The latter was the targeted method for this research project, but it was important to show that the primers work using the conventional detection methods as well.
- The RPA conditions and reagents were optimised for all 3 pathogens, to determine the most suited temperature, primer concentrations and DNA input when working with an “ideal” sample (DNA from pure, cultured microbes). Using these microbes, it was demonstrated that the RPA PCR method, a form of isothermal PCR, works well for all 3 pathogens.
- Three different methods of DNA extraction; a manual, column-based DNA extraction method, a manual magnetic bead isolation method and a modified, chemical DNA purification method using from the targeted bacteria, were investigated. Kits were evaluated for their cost, ease of use and infrastructure and additional materials needed. DNA was successfully extracted from all 3 organisms with the 3 different kits (Omega Biotec Mag-Bind® Environmental DNA 96 Kit (using magnetic bead purification); the Zymo Research Column-based purification with the Quick Faecal/Soil Microbe kit; a modified chemical extraction and clean-up using the Promega Wizard genomic DNA Purification kit).
- The limit of detection of pure DNA used in conventional PCR and RPA was determined for all 3 organisms using detection both with agarose gel electrophoreses and a commercial lateral flow device. The data indicate that, for pure DNA isolated from pure bacterial cultures, that picogram amounts of DNA can be

successfully amplified and detected with RPA PCR and the PCRD commercial lateral flow detection device.

CONCLUSIONS

In conclusion, it was demonstrated that an isothermal PCR method, namely recombinase polymerase amplification, can be useful to detect the presence of DNA extracted from cultured *Escherichia coli* 0157:H7, *Clostridium perfringens* and *Legionella pneumophila*. Further, it was showed that a commercially available lateral flow device can be successfully used for detecting PCR amplicons generated from nanogram amounts of DNA used for PCR. This indicates that low bacterial burden of these microbes potentially could be detected by using this specific PCR method and lateral flow device in environmental water samples, but further experimentation is needed to verify the impact of sampling volumes, removal of inhibitors in water, removal of extracellular DNA from non-viable/dead bacterial bioburden, and other steps where water are manipulated prior to DNA extraction. This specific PCR amplification method and lateral flow detection method thus far had not been reported in the literature for *C. perfringens.*

RECOMMENDATIONS

A backup plan must be considered, looking at other isothermal PCR methods as well. The reason for this is that limited companies are supplying commercial kits for different isothermal PCR. This might influence availability/supply of reagents if such providers close or remove products from their services.

Both conventional and isothermal PCR could be useful to detect pathogen DNA present in any samples to be tested AND can be detected by a commercially available lateral flow cassette. Diagnostic laboratories who intend to use this method at point of care, still will need a basic field laboratory fitted with at least a heating device, a microcentrifuge and at least a vortex device to assist with efficient bacterial lysis (notably for pathogens with cell envelopes more difficult to lyse). The latter is not always necessary but is known to improve DNA isolation. Also, extraction free methods to obtain DNA for PCR would be ideal for field work, but water of varied origin might present a challenge due to bioburden and potential presence of interfering chemicals. Hence laboratories must consider evaluating which water samples needs a DNA extraction kit and which samples might be fit to use with a simple boiling step and no extraction.

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ACRONYMS & ABBREVIATIONS

DNA	Deoxyribonucleic acid (DNA)
LFA	Lateral flow assay
PCR	Polymerase chain reactions
PG	Picogram
RPA	Recombinase polymerase amplification
RPA-LFA	Combination of recombinase polymerase amplification and lateral flow assay

GLOSSARY

Master mixes. A mix of all the ingredients needed for PCR, consisting usually of everything necessary to make new strands of DNA. The master mix usually contains everything, except the nucleic acid template and primers, which is added afterwards and depends on the target you want to amplify.

PCR. Polymerase chain reaction, a method whereby new DNA fragments are synthesised in a tube containing all the ingredients to make new strands of DNA.

Primer design. Method whereby the nucleotide composition of a DNA target is used to design short pieces of DNA (primers) that is identical to the beginning and end of the DNA target.
Fluorophore. A chemical compound that can re-emit light upon light excitation.

Primers. Very short, single stranded DNA fragments used to find a specific target on a larger piece of nucleic acid, is needed to initiate PCR reactions from the site where it binds.

Polymerase. An enzyme capable of building new strands of nucleic acid

Isothermal PCR. Meaning amplification of DNA is done at a single temperature, as opposed to traditional PCR where heat is used to separate double stranded DNA.

Amplification. Generating new DNA fragments synthetically in a tube, using a mixture of nucleotides, primers, enzymes, and a nucleic acid target.
RPA. Recombinase polymerase amplification

Lateral flow detection: a method where either a sample, or pure proteins or DNA or amplicons are labelled with specific compounds/ labels, then allowed to interact with antibodies bound to the lateral flow strip. Interaction results in either the development of colour or fluorescent, depending on the labels added onto the molecules being tested. This developed colour is visible with the naked eye. The answer usually is present/not present, and this is not a quantitative method.

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CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Water is an essential component for sustainable living, it supports households and various sectors that is vital for healthy economy. Water scarcity, overpopulation, drought, and increased pollution necessitates the repurposing of treated and reclaimed water (from sewage and other processes). Furthermore, the threat of pollution of reclaimed water used for recreation and irrigation demands proper screening of such repurposed water, to ensure it is fit for use and pose no threat to public health. The gold standard to monitor for microbial contamination of water is culturing of microbes, using coliforms as a reference point to indicate potential problems with water quality(*1*).

However, the use of repurposed water demands improved and rapid screening for potential residual, viable organisms as some microbes and parasites with spore-forming life stages are known to more resistant to chemical decontamination and potential useful alternative indicators (instead of coliforms)(*1, 2*), yet not part of routine screening in diagnostic environments. Furthermore, some local diagnostic laboratories doing routine screening are not yet equipped with staff and infrastructure to do culture-independent, molecular methods to detect microbial contamination in water. In addition, the use of expensive equipment or instruments requiring specific kits (frequently limited to be used only on specific instruments) is counterproductive in resource-poor settings. Ideal molecular methodology, at least for resource poor settings, could be to do isothermal PCR coupled with culture-independent detection of viability and lateral flow devices (*3, 4*).

The combination of abovementioned technology would enable point of care detection without using conventional PCR or real-time-PCR instruments. The latter requires constant electrical supply and PCR reagents that would enable detection of fluorescence. More studies are needed to evaluate the use of isothermal PCR technology. Amongst isothermal PCR methods, recombinase polymerase amplification is only method that can be done below 50°C with 2 studies even indicating that this method reliably amplify DNA targets with the use of human body heat (*5, 6*), thus without the use of a heated device.

This study was aimed to evaluating RPA and lateral flow detection of 3 pathogens relevant to water quality management. Not only is this method feasible to do with a batter-operated heating device, but the rapidness of this specific PCR method (amplicons are reportedly present within 15minutes vs standard PCR that takes at least an hour to complete) enables fast turnover times to test reporting. Furthermore, if the method is adapted to generate labelled amplicons, detection can be done using lateral flow devices, as increasingly reported in the published literature for various animal, human, and plant pathogens (*7-9*) . Incorporation of RPA into routine diagnostic, on-site testing relevant for use as mode of surveillance and ensuring potable water are free of pathogens thus could enable potential on-site sample testing. The use of isothermal PCR combined with Lateral Flow Assay (LFA) methodology will enable molecular detection of pathogens in water without the use of real-time PCR equipment. Ideally, this method should be combined with confirmation of bacterial viability; the latter is done by incorporating chemicals such as PMA (or similar molecules) into PCR reactions. PMA enables discernment of DNA from viable microorganisms and exclude the amplification of residual DNA from dead cells(*10*).

1.2 MICROBIAL WATER QUALITY MONITORING

Sustainable Goal 6 of the United Nations envisions clean water and proper sanitation globally (United Nations, no date), The UN predicts a water crisis by the year 2040, with a predicted 40% shortfall in freshwater. The organisation encourages pro-activity by local governments to prevent the predicted crises (Water action decade, no date)

Water is an essential resource for a sustainable economy and healthy living, but rapid population growth and increased industrialisation places an increased pressure on natural resources such as land and water. Increased pollution of water bodies, including contamination with human pathogens, further impacts water safety. Improved and rapid detection of pathogens in water is becoming increasingly important as water scarcity increases. Lack of safe drinking water, as well as poor surveillance of environmental waters contributes to diseases in individuals exposed to contaminated water. Therefore, improved, and rapid surveillance are needed, ideally molecular methods combined with confirmation of viable micro-organisms detected by molecular methods.

Water scarcity and adequate supply to an increasing population requires the use of alternative safe, potable water sources. Reclaimed sewage water is an attractive alternative to use in both industry and for human consumption(*11*). However, reclaimed water, if not properly monitored and quality controlled, might contain residues of viable, resilient microbes, particularly spore-forming bacteria that are more resistant to chemical disinfectants. As such, pathogens with resistant life stages (such as spores), poses a threat to human health; even more in countries with high burden of conditions predisposing vulnerable persons to opportunistic infections. Resilient bacteria (such as clostridia, *Legionella*) and protozoa (such as *Cryptosporidium*, *Giardia*) poses a public health threat and are found increasingly in higher numbers in water bodies. Thus, their potential presence in reclaimed water and other water containing bodies must be continuously monitored, especially those resources reclaimed for human and animal use (including recreation) (*12-14*).

The presence of one resilient organism can also be indicative of other resilient organisms present. For example, research noted an association of *C. perfringens* with *Cryptosporidium*, hence the detection of the former is advised to be used as an indicator of the potential presence of the other(*15*). As *Cryptosporidium* are more resistant to chlorination, and a cause of diarrhoea amongst vulnerable populations, monitoring of this micro-organism in waterbodies is vital(*16-19*). It had been noted previously, that microbes that are more resistant to chemical disinfection (compared to coliform bacteria), could be better indicators to monitor or detect sub-optimal sewage treatment processes, such as that found in aging sewage plants(*20*). Microbes resistant to sewage chemical treatment is a particular concern for reclaimed waiter, as the presence of residual, viable pathogens in reclaimed water thus must be excluded if to be safely repurposed for human use. The use of reclaimed water as supplement potable water supplies increases the risk of microbial infection and thus should be monitored appropriately(*21*).

Spore-forming bacteria and parasites with varied life stages are a particular headache for water quality control as that enables their survival in the environment for extensive periods For example, it has been observed that spores of *C. perfringens* accumulate in sediments below water bodies, thus a source from where it can seed water (*22*). This has implications for waterbodies used for recreation or used for agriculture.

1.3 PATHOGEN DETECTION METHODS

1.3.1 Culture-based methods

Culture-based detection of microbes remains a mainstay of water quality surveillance, particularly the detection of viable coliforms as a quality indicator of faecal contamination. However, as there are increasing evidence of treatment-resistant micro-organisms and drug resistant pathogens (or evidence of drug resistance genetic determinants such as plasmids) in sewage and water bodies (where treated effluents are released into), it is imperative to have improved, rapid monitoring for detecting pathogens in treated water (*23*). Time-

consuming and expensive culturing to enrich for and identify some of these human pathogens might not be feasible; more rapid, alternative detection methods is hypothesised to be useful. Specifically, rapid, culture-independent methods are increasingly eyed as viable possibilities to do surveillance, while limiting exposure time of humans to cultured pathogens from potentially contaminated waters. Ideally, closed systems using culture-independent methods would be ideal, though currently this ideal had not been achieved yet. With the current pandemic, numerous countries are using Polymerase Chain Reaction (PCR) to do surveillance of the SARS-CoV-2 virus in sewage and other waterbodies; the data gathered are useful to guide public health responses (*24*). Locally such surveillance is done by South African Medical Research Council wastewater Surveillance and Research Programme, who has an online, interactive dashboard with data (https://www.samrc.ac.za/wbe/).

1.3.2 Detection of pathogen nucleic acids

Detection of pathogen nucleic acids is a rapid method to identify micro-organisms (compared to culture-based phenotyping), although this method is hampered by the availability of technology and expertise in low-and-middle-income countries (LMICs). The impact of human, financial and other resources on the ability to deliver rapid service are well illustrated in recent publications reporting on setting up molecular services for SARS-CoV-2 surveillance on the African continent (*25, 26*). The lessons learnt from the COVID pandemic is useful to evolve towards more effective and rapid screening of pathogens, also in the context of pathogen surveillance in water. Numerous methods exist to amplify and detect nucleic acids (NAs). The original conventional polymerase chain reaction (PCR) combined with agarose gel electrophoreses and with/without Sanger sequencing are useful to identify microbes, but Sanger sequencing will delay pathogen identification with at least one week, especially if an external service provider is used. In this regard, specific detection of fluorescently labelled DNA amplified using real-time PCR and bacterial specific probes could provide rapid identification within 2-3 hours (inclusive of DNA extractions). The original, conventional PCR method and real-time PCR uses high temperature (>90°C) to separate double stranded DNA, followed by amplification of DNA at a lower temperature (68-72°C). This requires specialised instruments built to cycle at different temperatures, sensors to detect fluorescent amplicons in the case of real-time PCR.

Most importantly, a stable electricity source. Numerous studies have reported on the diagnostic utility of real-time PCR as a tool enabling rapid identification of various pathogens. Importantly, and depending on the specific instrument, PCRs can be multiplexed to detect more than one pathogen in a single tube/PCR run. An example of its utility to screen water was demonstrated in a 2017 outbreak of *Cryptosporidium spp*. amongst military service members in a camp, with real-time PCR identification of the pathogen in the patients and the potable water, enabling public response whereby the water sources were treated appropriately to provide safe, potable water to the facility (*27*). Limiting factors for the use of real-time is the availability of instruments, staff proficiency, stable provision of electricity and backup generators (to prevent interrupted runs), availability and cost of supplies. Therefore, alternative molecular methods are increasingly attractive as diagnostic options.

Isothermal PCR, a method done at a single temperature and excluding the need for expensive thermal cyclers, are an attractive alternative that is increasingly used. Isothermal PCR methods can be done as a conventional method (meaning amplicons detected using agarose gel electrophoreses or lateral flow technology) or could be done as a real-time PCR method where amplicons are visualised through fluorophores (*28-31*). A recent paper illustrated the impact of resource and infrastructure limitations on the ability to do molecular surveillance, specifically reporting the importance of incompatible equipment (real Time PCR instruments should be purchased with consideration of the type of locally available fluorophores that can be “read” on the specific machine, also should consider the kits to be used), limited skills of available staff (therefore training is an essential component), unstable (unreliable) electric power supply (to enable uninterrupted PCR runs and timely outputs), an effective procurement and supply chain, and lastly overhead costs.

As mentioned before, isothermal PCR (done at a single, lower temperature) excludes the need for expensive PCR equipment. Instead, a heating block or water bath at one temperature are sufficient for amplification to occur. Products from isothermal PCR are detected using gel electrophoreses or monitoring of fluorescence in real-time as amplicons are produced. The first developed isothermal PCR method, loop-mediated amplification (LAMP), had problems with specificity and therefore still is not a preferred diagnostic method on its own. Other

isothermal methods, namely helicase dependent amplification (HDA) and recombinase polymerase amplification (RPA) are more specific compared to LAMP. Helicase dependent amplification (HDA) or recombinase polymerase amplification (RPA) yields more sensitive and specific isothermal reactions, and these two methods are increasingly used in commercial platforms such as the Solana instrument (https://www.quidel.com/molecular-diagnostics/helicase-dependent-amplification-tests) or in-house developed diagnostic assays on any open platform thermal cycling device. RPA technology has already been successfully used to identify *Legionella* and *Enterococcus faecalis* in water (https://www.twistdx.co.uk/en/innovation/water-testing*(32)*) and various other pathogens.

A factor improving the utility of isothermal PCR, is the ability to detect amplicons using lateral flow devices, thereby excluding gel electrophoreses and UV transillumination to detect amplicons. RPA (and other isothermally produced) amplicons can also be visualised by simply mixing amplicons and SYBR-I fluorescent dye, followed by exposure to ultraviolet light at 395 nm (*33*).However, this method could be subjective and varied between analysts.

The combination of isothermal PCR with lateral flow assays enables point of care detection of pathogens; this combination depends only on the availability of reagents and a heated source. LFA technology to detect amplified DNA (hereafter NALFIAs) use conjugated particles immobilised on a paper strip (gold labelled nanoparticles or carbon particles embedded onto nitrocellulose are used by most manufacturers). Detection on strips is via colour development on the strip, e.g. if a sample contains relevant amplified, labelled targets, it first binds to conjugated particles as it flows across a flow strip, the newly formed complex will then interact with an immobilised antibody target complementary to the label on the one end of the amplicon. Colour development is facilitated via streptavidin-based chemistry; upon binding and relevant chemical reactions, a visible coloured line confirms the presence of targets. This assay demands well designed primers, but have been employed successfully in the food, medical and other industries to detect microbes in varied sources. It requires extensive optimisation of primers for isothermal PCR, especially if DNA from complicated samples is to be tested.

Lateral flow strips can be assembled in-house (from assembly of individual ingredients and in-house printing of test lines on strips), or custom-made strips can be bought from suppliers such as TwistDX (https://www.twistdx.co.uk/) or Quidel (https://www.quidel.com/molecular-diagnostics/helicase-dependent-amplification-tests) or Abingdon health (the PCRD device, Abingdon, UK). The use of NALFIA improves on the non-specificity of amplification observed with isothermal amplification methods and already successfully to detect various pathogens in different studies, provided PCR are adequately optimised (*34-38*). Notably, the technology is useful to detect microbes of clinical importance (*39*). Of course, real-time isothermal PCR would enable proper quantitation of nucleic acid targets, as opposed to LFA strips which would be semiquantitative and potentially more subjective. However, LFA enables detection is ideal for low-income settings or remote places with no or irregular electrical supply, especially in the absence of other available technology. Heating devices, for amplification, could be battery operated or have a mobile unit supplying electricity, enabling heating of an instrument as well as the use of at least a water filtering device (to concentrate microbes on a filter) and a mini centrifuge for nucleic acid extractions from water samples.

To summarise, commercial platforms are extremely convenient to use, but low-and-middle income countries (LMICs) are limited by financial and infrastructure constraints. Furthermore, one frequently is limited to use assays specifically developed for such commercial platforms. For that reason, development of local expertise to develop in-house assays is imperative, enabling design of in-house assays necessary for local needs. Assays that are easy to perform, quick and excludes the requirement for expensive detection equipment (such as real-time PCR machines) is also more practical in low-resource settings. Finally NALFIA could be a solution to detect microbes not routinely surveyed for water quality management or where quick/urgent detection is necessary, as test results are available within an hour after DNA extraction. It could be adapted to detect any pathogen, whether bacteria, viruses, fungi, or protozoa. Thus far, limited studies looked at the utility of this technology for water quality management. In the study proposed here, nucleic acid amplification combined

with lateral flow immune detection assay (NALFIA) method to detect pathogens was optimised and employed to detect two resilient microbes (*C. perfringens*, *Legionella pneumophila*) as well as a less resilient, but equally important pathogen (*E. coli 0157:H7*). Pure cultures were used for validation of the method, and limited number of water samples were tested with this method.

The methods itself could be useful for local monitoring, but diagnostic laboratories should do their own validation, to ensure the DNA extraction methods employed in the specific facility, combined with the sample types and the specific amplification method employed is optimal to provide reproducible, reliable, sensitive, specific and quality results. Importantly, the skills transfer in this study will grow local expertise, which can be ploughed back into staff doing routine diagnostics and not yet equipped to do molecular work.

1.4 PROJECT AIMS

The aims of the project were:

1. To develop a rapid molecular method to detect *C. perfringens*, *L. pneumophila* and *E. coli* using recombinase polymerase amplification and paper based lateral flow assay.
2. To test optimized method with various water resources (drinking water, pre- and post- sewage treatment effluents, outfalls, various water environments in Cape Town monitored by local authority).
3. To confirm viability of the target bacteria in the tested water samples, using standard culture methods and media to isolate these bacteria.
4. To do cost comparison of molecular versus culture-based methods in terms of human and laboratory infrastructure and consumable resources required.

1.5 SCOPE AND LIMITATIONS

1.5.1 Scope of project

A commercially available isothermal PCR kit was used to optimise a specific isothermal PCR method. The specific isothermal method is recombinase polymerase amplification, where a DNA recombinase is used to separate double stranded DNA. For this study, a thermal cycler was used to optimise the method for the 3 bacterial targets. Purchased DNA of the targeted bacteria (*C. perfringens, L. pneumophila* and a Shiga toxin negative *E. coli* 0157:H7 strain) were used as templates for the PCR to be optimised. Previously described primers, targeting genes unique to *L. pneumophila* serogroup *E. coli* 0157:H7, was validated, while new primers were designed for *C. perfringens.* For lateral flow detection, the reverse primer of each primer set was labelled with biotin, while the forward primers for *C. perfringens and L. pneumophila* was labelled with DIG and he forward primer for *E. coli* labelled with 5-FAM. This enabled generation of labelled amplicons to be captured on a commercial lateral flow device and detection of amplicons with streptavidin-biotin chemistry. The sensitivity of the method was determined by serially diluting pure DNA of the respective microbes (range 10 ng to 1x 10^{-6} ng) and using the dilution series as input templates for both conventional and isothermal PCR to discern the lowest amount of input DNA that will generate visible amplicons on agarose gels and on lateral flow strips.

Finally, the RPA-LFA detection method using DNA extracted from filtered water samples. DNA was extracted using a column-based DNA purification extraction kit that contained a step to remove PCR inhibitors from water samples. Three samples collected and provided by the Scientific Services Division (City of Cape Town), representing different sample types (and potential PCR inhibitors) were tested for the presence of the 3 pathogens (marine water (from a tidal pool), post-treatment water and industrial effluent). These samples respectively had a low, low, and high bioburden (respectively), impacting the amount of water that could be filtered. Nevertheless, the presence of DNA from the relevant pathogens were demonstrated using the RPA method and lateral flow detection, providing proof of principle that the methodology is fit for purpose.

1.5.2 Limitations of the methodology

Molecular detection of microbes does not equate to live, viable microbes in a tested sample. For viability, samples could be filtered and stained with specific dyes (e.g. PMA (henanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride) to distinguish between live and dead bacteria. Also, culturing of target microbes could be done, although it must be noted that microbes could be viable, but non-culturable (thus culturing might not optimally detect the presence of bacterial pathogens. Culturing is also cumbersome. In the case of *C. perfringens,* a laboratory must culture under anaerobic conditions (implying additional costs for anaerobic sachets and equipment, or specific gases and an expensive anaerobic workstation). **Future/follow-up studies should optimise the PCR in the presence of PMA, to do so called "viability PCR", thereby excluding culturing. This needs to be optimised with various water sample types as the impact of contaminating biomolecules and other chemicals needs to be assessed.**

No culturing was done from the various sample types. Reason: Culturing from environmental samples runs the risk to culture microbes of animal and bird origin. Although one could use selective media to enrich for specific microbes, culturing from the environment could also yield other, undesirable pathogens (that requires more than Biosafety Level2 laboratory), therefore it was decided to rather explore the viability PCR option and not culturing.

This specific project only aimed to evaluate and optimise the isothermal RPA method; thus, our results only show proof of principle that as little as picomole amounts of nucleic acid in a system will be successfully amplified via this method. While the water samples tested positive for relevant pathogens, the PCR presented here is not proof of viability. For that, further optimisation should be done with RPA in the presence of reagents such as PMA (henanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride) to ensure amplicons are generated from viable microbes and not residual DNA from dead microbes. This will be the scope of future work.

Lastly, at the time of project development, we initially aimed to use a specific commercial, ready to use kit (from company TwistDX) that enables generation of PCR products ready for LFA detection and included the use of a DNA probe. This kit is not available on market any longer, thus not possible to use. The approach was thus changed to generate dual labelled amplicons via a different RPA KIT (from same manufacturer) to detect on a commercially available LFA strip. These strips are pre-manufactured to capture DIG or 5-FAM labelled amplicons on a nitrocellulose paper strip imprinted with relevant antibodies to capture the abovementioned molecules, followed by streptavidin-based chemistry to visualise captured amplicons. HOWEVER: although the negative controls used shows the absence of false positive tests, more validation is necessary to ensure test specificity. For *L. pneumophila* and *E. coli* 0157:H7, the primers used were validated extensively before, but the phospholipase C primers (*C. perfringens* specific gene), should be validated more.

This study only tested a limited number of water samples (one from different origins each). To ensure the use of a fully validated test, it is advised that much larger set of samples, with repeated sampling from the same environment are tested. This will enable appropriate statistical analysis, as well as would inform on the impact of long-term monitoring of a specific environment using molecular detection of pathogens. Such data could inform on the frequency that sampling is needed (especially in the vicinity of fallouts or where industrial effluent contaminate natural environments), cost, feasibility and comparison with routine microbial culturing. The latter specifically will enable the generation of comparative data for coliform surveillance as indicator of contamination versus alternative indicators such as *C. perfringens.*

A major limitation of RPA methodology is that currently only one company supply ready-to-use RPA master mixes. As the principle of the method is known, local research potential local biotechnology experts could explore the use of other recombinase enzymes and the development of locally supplied kits. One could purchase individual ingredients and optimise the complete RPA assay in-house; this was not within the scope of this study.

Lastly: this study used pure, extracted DNA to evaluate the RPA method. The ideal point-of-care test involves no DNA extraction, rather using crudely lysed microbial cells and no purification, or a closed device where extraction and PCR occurs in a closed module. For example, one study reported that diagnostic turnover time was diminished when SARS_COV2 PCRs were done using crude, boiled respiratory human diagnostic samples (method briefly involves resuspension of swabs in relevant buffer, boiling of resuspended material for a brief period followed by PCR setup). While this potentially is feasible for filtered and concentrated low bioburden, less complicated samples (for example water housed in taps and sandpipes), this might not be feasible for water samples with a high bioburden of polymicrobial origin. Nevertheless, where RPA PCR could be done without crude DNA, it will drastically diminish cost of testing and time to diagnostic outputs. This was not explored in this study, but should be investigated in future studies, notably as some kits on the market claim the capability to generate “extraction-free, fit for PCR” DNA, while using minimal instrumentation (a feasible option for resource poor environments, if valid and reliable to use).

CHAPTER 2: EVALUATION OF DIFFERENT DNA EXTRACTION METHODS FOR THE DETECTION OF PATHOGENS

2.1 INTRODUCTION

For this study, DNA extractions using locally available commercial kits (specifically those not previously reported in local literature for use with water) was explored, although only pure cultures were evaluated at the time of this final report. Due to eventual time constraints, kits were not evaluated yet for use with real world samples. This also requires extensive testing, as a proper validation requires sufficient replicate extractions per specific sample (and testing different sample types) to generate quality data with appropriate statistical outputs, verifying that reliable results are obtained with specific standard operating procedures. Therefore, only DNA extraction from pure cultures is reported here. A literature search was done initially, to understand which DNA extraction kits had been reported, in peer reviewed literature, as good options to extract DNA from environmental waters. While some kits are more readily used and reported on (for example using the DNEasy Powerwater kit (Qiagen) are a popular choice and widely reported on), yet other options are available on the local market. Many are not yet evaluated /validated for DNA extraction from water or only reported in limited studies. For this study, the MagBind Environmental DNA 96 kit (Omega Biotek, kit 1), Quick-DNA Bacterial/Fungal kit (Zymo Research, kit 2), and the Wizard Genomic DNA Purification kit (Promega, kit 3) were evaluated for the ability to extract DNA first from pure bacterial cultures with known input of colony numbers. At the time of submission of this report, no peer reviewed publications, using these kits for extraction from water, were found. These kits are fairly competitive in pricing, ranging between R5 000-10 000 per 50 samples, thus reasonable.

2.2 DNA EXTRACTION METHODS

These kits represent 3 modes of DNA extraction (Figure 2-1), with kit 1 using magnetic bead purification, kit 2 using a solid matrix (or column based) purification, and kit 3 using a modified chemical method where DNA are precipitated in the presence of alcohol, air-dried and rehydrated in TE or water.

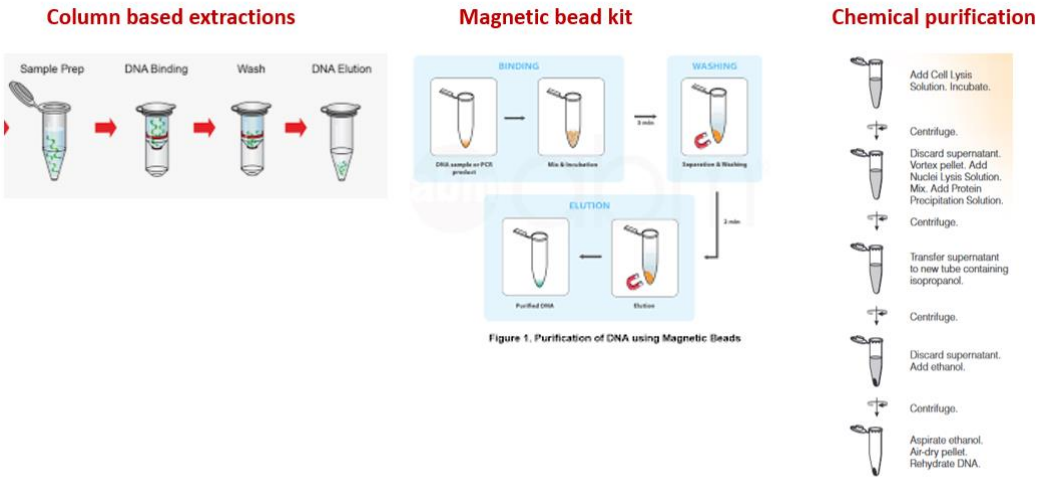

Figure 2-1: Different kits used for DNA extraction in this study

2.3 SUMMARY OF RESULTS

As it is easy to obtain sufficient DNA from >10^8 bacterial cells, this study tested first on obtaining sufficient DNA with input cells of 10^9 and 10^8 starting material of the targeted bacteria. For this, colonies from overnight bacterial cultures were resuspended in phosphate buffered saline, standardised to McFarland standard of 0.5 (i.e. approximately 10^9 colony forming units per ml of bacteria) using the VITEK Densicheck instrument (BioMerieux, France) for standardisation. These cell suspensions were used to prepare serial dilutions of cells in PBS, whereafter 1ml aliquots were prepared, centrifuged and frozen until used for DNA extractions. That was done to ensure that the same number of cells were used for extractions, enabling appropriate comparison. An aliquot of the serial dilutions was used to plate on culture media, grown overnight and bacterial colonies counted to confirm cell numbers per dilution (data not shown).

Nevertheless, it was clear that cell pellets were invisible for 10^7 cells and below, thus extraction from this dilution and less cells were abandoned after initial extractions. Notably, the amount of DNA from these cells also was not detectable using a Nanodrop or Biodrop instrument, meaning scientifically we could not verify the amount of DNA obtained from such low cell numbers from the kits or in fact if DNA was present. Potentially one could do PCR to discern if DNA is amplified, but a negative PCR in this instance does not indicate whether DNA was successfully extracted (if reading is too low for laboratory instruments), or if one in fact lost cell pellets during the processing steps.

Secondly, the volume that extracted DNA (prepared from 10^9 and 10^8 cells) were eluted in half the volume of what the kit instructions prescribed. Again, this was to ensure that DNA concentration was high enough to be detectable using laboratory instruments such as a Nanodrop or Biodrop, and purity could be confirmed at least using the Nanodrop and Biodrop instruments. Obtaining values is important when one needs to compare yield and potential purity. Also, one could do proper limit of detection studies if you have a verified amount of DNA as starting material. This specific observation is relevant for when one has very low numbers of a specific bacterial target in a sample, as too low numbers might yield too little DNA template that might not amplify sufficiently (thus generating a false negative result), especially in the presence of competing DNA from other sources or inhibitors. Also, one could roughly correlate the DNA concentration to the number of bacterial cells, if sufficient replicates of cell counts are done (for statistical calculations and confidence testing) and DNA extracted from the same sample with specific cell numbers (for an example, see (*46*)).

A potential solution is also to add a quality control, at a standard concentration, to samples to be extracted. This could serve as a DNA extraction control and amplification inhibition control, as it should be amplified in the same reaction mixture where amplification of the actual target is tested for. This extraction control and the bacterial target should be amplified in the same tube to discern if the competition for nucleotides in the amplification reaction would influence the lower limit of detection of the bacterial pathogen.

To date, DNA extractions, using 10^9 and 10^8 cells, were done in triplicate, whereafter DNA was quantitated using the Nanodrop 2000 Instrument (Thermos Scientific). Overall, for all 3 microbes, extractions from starting material of 10^9 bacterial cells, yielded DNA between 15-40 ng/ ul when eluted in maximum 50 µl of elution buffer.
This evaluation was done using pure bacterial cultures yet needs to be done using real world water samples, testing sufficient replicates from the same sample to ensure observations are reliable and reproducible. The LOD for real world samples might also differ between different sample types, thus observed LOD for one sample should not be extrapolated to other water sample types. Extractions with pure microbial growth was not explored further as actual validations should be done with real world samples, a focus of future work to conclude this project even after submission of this report.

With regards to the ease of use of the 3 different kits, it was observed that kits 2 (column based) and 3 (modified chemical and precipitation method) had the least steps to be done to obtain pure DNA and was very easy to

do. In contrast, kit 1 had more steps, including multiple and extensive vortex steps. Other than that, this kit was also easy to use and yielded good quality DNA from pure bacterial cells.

CHAPTER 3: OPTIMISATION OF RECOMBINASE POLYMERASE AMPLIFICATION-LATERAL FLOW DETECTION OF PATHOGENS

3.1 INTRODUCTION

PCR detection methods are useful also to do surveillance of microbes not traditionally tested in laboratories doing water quality monitoring. However, the availability of infrastructure and appropriate equipment might be a hindrance to do some PCR methods. Conventional and real-time PCR requires specialised equipment that do a typical PCR cycle of double stranded DNA separation at very high temperature (94-95°C), then allowing binding of DNA primers at much lower temperature (45-60°C), followed by synthesis of a new DNA strand at 72°C. This cycle needs to be repeated between 25-45 times, with swift modification of the metal block temperature. A complete PCR run using the abovementioned method requires about 1.5-3 hours to make DNA fragments below 5 kilobases in length. Notably, it requires an instrument capable of thermal cycling. In contrast, isothermal PCR can be done at a single temperature and employs proteins to separate DNA strands and keeping the DNA template single stranded.

Of these, recombinase polymerase amplification, or RPA, is an increasingly popular method whereby a DNA target fragments is generated at a single temperature (between0 37-42°C) using a mixture containing a recombinase to do strand invasion (break the double strand bonds, single-stranded DNA binding protein (to keep DNA single stranded), a polymerase to synthesise new DNA, nucleotides, relevant buffer, and the target DNA template. The primers (on average about 35bp long) binds to the recombinase and is guided by the recombinase to the specific DNA target. This nucleoprotein complex then finds the homologous genomic DNA target of the primers, strand invasion occurs, SSB keeps the DNA single stranded, and the polymerase synthesise new fragments. Amplicons can be detected by simply mixing amplicons with a fluorophore such as SYBR-1 and visual inspection under UV light, or with real-time PCR if primers are labelled on one end with a fluorophore such as 5-FAM or HEX or CY5. Ideal for field work, it can also be detected on paper-based devised/ lateral flow strips if products are labelled with digoxigenin (DIG) and a fluorophore such as FAM or FTC on the other end.

This method is significantly more rapid than conventional PCR (1-3 hours), or real-time PCR (on average 1 - 1.5 hours). The major feature of this method is that the method can be done using only a heated source or even body heat (*33*) or a heating block. Temperature required is lower than other PCR methods and ideally not above 45°C. An attractive option for point of care detection, is the potential to do amplicon detection using lateral flow detection and paper-based devices. The widespread availability of commercially produced, ready-to-use LFA strips and devices have led to increased use of amplicon detection using LFA, very useful to do studies in the field or point-of-care detection in low-resource settings.

3.2 OPTIMISATION OF PCR CONDITIONS USING CONVENTIONAL PCR

3.2.1 Bacterial strains

Quality control reference isolates were purchased from Microbiologics (https://www.microbiologics.com/, St. Cloud, MN, USA). DNA was extracted from cultured isolates and used for PCR amplification. The strains are:

1. *Legionella pneumophila subsp. pneumophila* derived from ATCC® 33152™*, catalogue No. 0211P.
2. *Clostridium perfringens* derived from ATCC® 12915™, catalos No. 0801P (this strain is *plc* and *cpe*+, thus toxin type F, known to cause non-invasive, self-limiting diarrhoea.
3. *Escherichia coli* (O157:H7) derived from ATCC® 43888™, No. 0795P. Biosafety Level 1, as this strain is Shiga toxin negative and used for quality control in diagnostic laboratories).

3.2.2 Culturing

Cultures of *E. coli* and *C. perfringens* were prepared on purchased 2% Brucella sheep blood agar (Greenpoint media), while *L. pneumophila* was cultured on BCYE agar amended with BCYE agar amended with glycine, vancomycin, polymyxin B, cycloheximide (GVPC). Additionally, *C. perfringens* were cultured anaerobically, using AnaeroPack sachets and anaerobic jars (Thermo Scientific™ AnaeroPack™ 2.5L Rectangular Jar). For optimisation of PCR, cells were harvested from plates and extracted using the Quick-DNA Fungal/Bacteria miniprep kits (https://zymoresearch.eu/collections/quick-dna-fungal-bacterial-kits, Zymo Research).

3.2.3 Primers and PCR

Primers for *C. perfringens* phospholipase C gene were designed as per TwistDX guidelines (https://www.twistdx.co.uk/rpa/using-pcr-primers/), using free, online software Primer 3 (https://primer3.ut.ee/). Candidate primers were tested for the ability to bind only *C. perfringens* DNA, by BLAST analysis (https://blast.ncbi.nlm.nih.gov), checking it against all available microbial genomes in this free, online database. Following this, the most ideal pair predicted by Primer3 were ordered. For *L. pneumophila (40)* and *E. coli (41),* previously described RPA primers were used. Primer sequences are reported in Table 3-1. Synthesised primers were reconstituted with 1xTris-EDTA Buffer (10 mM Tris,1 mM EDTA, pH 8.0), preparing stocks of 100 µM. For PCRs, these were diluted to 10 µM with molecular grade water and used as the working stocks for PCR. All primers were tested first with conventional PCR to verify it amplifies the correct size product, before the TwistAmp™ Basic system was used for RPA. After confirmation that the correct size amplicons were generated, primers modified with biotin and digoxinin (DIG) labels or 5-FAM, were used, again checked that it generates amplicons of the correct size. Reverse primers for all three organisms were modified with biotin, while the forward primers were labelled with DIG (for *C. perfringens plc* and *L. pneumophila mip* genes) and 5-FAM (for *E. coli* 0157:H7 *rfbE* gene). These labelled primers were purchased from Integrated DNA Technologies (IDT, https://eu.idtdna.com/pages/products/custom-dna-rna/dna-oligos).

Table 3-1: Primers used in this study.

Gene targeted	Organism used	Primers (5'-3' direction)	Reference
16S RNA	All bacteria	Forward: AGAGTTTGATCCTGGCTCAG Reverse: AAGGAGGTGATCCAGCCGCA'	(*42*)
plc	*C. perfringens*	TTAGCTTATTCTATACCTGACACAGGGGAATC GCAGTAACATTAGCAGGATGATATGGAGTA	This study
mip	*L. pneumophila* serogroup1	GACAAGGATAAGTTGTCTTATAGCATTGGTGC CTTGTTAAGAACGTCTTTCATTTGCTGTTCGG	*(40)*
rfbE	*E. coli* 0157:H7	AGCTTTGTTAGCGTTAGGTATATCGGAAGGAGA ACATGGATGTCCGTATAAATGGACACACATAAT	*(40)*

3.2.4 Conventional and recombinase polymerase amplification of targets

The identity of purchased reference isolates were confirmed by doing 16s ribosomal RNA PCR and Sanger sequencing. For the primer sets used for RPA assays: To verify that the correct DNA target was amplified,

conventional PCR was done first, amplicons size verified by agarose gel electrophoreses, whereafter the nucleotide composition was determined by Sanger sequencing (done by service provider Inqaba Biotech (Pretoria, ZA). Quality assessment of generated DNA sequences was done using relevant software such as Chromas (Technylesium, Australia), followed by verification of identity and the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Conventional PCR reactions typically contained primers at final concentration of 0.5 µM, 1 µl of dNTP mix (10 mM each stock), 3 µl of $MgCl_2$ (25 mM) was done using either the GoTaq system (Cat nr M300, Promega) or the GoTaq Green Master (M7122, Promega). Water and buffer were added as per the specific kit instructions. PCRs were done in the GeneAmp 2700 PCR System (Applied Biosystems). Typically, 20-50 ng of DNA or bacterial colonies were used for PCRs. The cycling parameters, for all 3 primer sets, were typically: initial denaturation for 5 minutes at 95°C, then 35 cycles consisting of DNA denaturing for 30 seconds at 94°C, primer annealing for 30 seconds at temperature ranging from 55- 64 ° C (dependent on the PCR), amplification for 30 seconds at 72°C. A final extension was done 5 minutes at 72°C.

For **RPA amplification,** reactions were carried out as guided by the protocols for the TwistAmp® Basic kit (TwistDx, UK). Initially, the TwistAmp® Liquid Basic kit were used, but subsequent work was done using the lyophilised, TwistAmp® Basic kit. For the latter, rehydration buffer, molecular grade H_2O were added to dissolve the dry enzyme pellet, whereafter forward and reverse primers and DNA template was added. The reactions were initiated by the addition of magnesium acetate was added to the cap of the tube. PCR reactions were done in a total volume of 50 µl, at 37°C in a thermal cycler (Applied Biosystems) for 30 min, whereafter reactions were analysed using gel electrophoreses and detection on lateral flow strips. Initially, temperature optimisation was done, but all RPAs worked at 37°C, therefore this temperature was used for the RPAs results reported here.

3.2.5 Detection of amplicons

3.2.5.1 Gel electrophoresis verify size and confirmation of ID using Sanger sequencing.

Agarose gel electrophoresis was done, using 2% agarose gels submerged in 1x Tris-Acetate-EDTA buffer (diluted with water from 10x TAE, product number T9650, Sigma Aldrich). DNA Molecular weight standards will be run on same gels, to verify the size of amplified PCR products. Gels were run for 1 hour at 120V. Amplicons were visualised using the Biorad Geldoc Imaging System (Biorad).

3.2.5.2 Detection using lateral flow analysis (LFA)

Amplified, labelled PCR products were analysed on the PCR device as per manufacturer instruction (Abingdon, UK, https://www.abingdonhealth.com/products/pcrd/). Typically,6 µl of amplicons were mixed with 84 µl of the buffer provided by the manufacturer. An aliquot of this (75 µl) was loaded onto the device as per instructions, colour development visually inspected for a maximum period of ten minutes and recorded by photography on a mobile device. Ma and co-workers, whereby they labelled RPA amplicons using biotin and DIG and successfully detected a viral target using LFA strips (*43*).

3.3 RESULTS

3.3.1 Confirmation of identity of purchased isolates

After culturing from purchased culture material, the identity of the purchased reference isolates was confirmed, via Sanger sequencing as indeed *C. perfringens*, *L. pneumophila* and *E. coli* 0157:H7. DNA was extracted

(for the purpose of PCR optimisation) and eluates ranged from 15-50 ng/µl, as confirmed using a BiodropDuo instruments (https://biochrom.co.uk//brand/23/biodrop.html).

3.3.2 Validation of newly designed *plc* primers

To validate that the newly designed primers for *C. perfringens* were specific, generated the right size amplicon and also the optimal temperature and primers to use, conventional PCR was done. The results showed that the primers worked well, generating the amplicon of expected size (DNA template input range was 5-40 ng) (Figure 3-1). Sanger sequence and BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed that an amplicon of correct identity was generated via for conventional PCR (data not shown). Primers for *E. coli* and *Legionella* were not verified with conventional PCR, as these are published primers and previous validations published (*40, 41*).

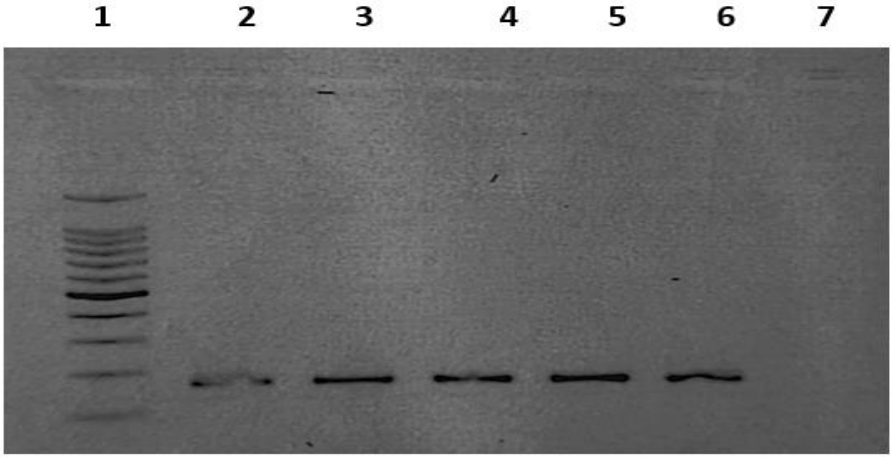

Figure 3-1: Confirmation of *plc* internal fragment amplification. Agarose gel electrophoresis showing that the designed primer pair successfully generated amplicons of correct size, with no non-specific amplification. Lane 1: DNA size marker (Generuler 100 bp ladder), Lanes 2-5: *C. perfringens* amplicons generated with input DNA of 5,10, 20,40 ng of DNA, Lane 6: PCR input DNA 55 ng, Lane 7: negative control (water). Amplicons were generated using GoTaq Mastermix and primer hybridisation temperature were 64°C.

3.3.3 RPA amplification and optimisation

Following confirmation that the *plc* primer set amplified the correct target, RPA PCR was attempted. The protocol was followed as per the TwistAmp Liquid Basic kit, multiple reactions were set up and incubated at increasing temperatures, to discern the optimal temperature to use. The result showed that 37°C was applicable to amplify the *C. perfringens plc* gene fragment, as non-specific amplification was observed at higher temperatures. A faint non-specific band was visible for the lowest temperature tested as well, but this was less than other lanes (Figure 3-2). The *plc* RPA mixture was done as per the TwistAmp Basic protocol, with no modification of any reagents. Only the optimal temperature was verified (Figure 3-2). This PCR potentially could be further optimised (perhaps do primer titration or the amount of Magnesium acetate titration), to remove the non-specific band seen when PCR was done at 37°C. The amplicon was sequenced and confirmed to be originating from *plc*, as expected (data not shown). RPA PCRs were done for *L. pneumophila mip* gene and the *E. coli* 0157:H7 *rfbE* gene, using the basic setup of the TwistAmp Basic protocol. These genes are unique to the specific strains and thus markers for their presence, discriminating these pathogens from other strains or serogroups from the same species. Primer titrations as well as temperature titrations were done. For *rfbE*, amplicons were observed at 37-39°C, also with some non-specific bigger size bands (Figure 3-3.)

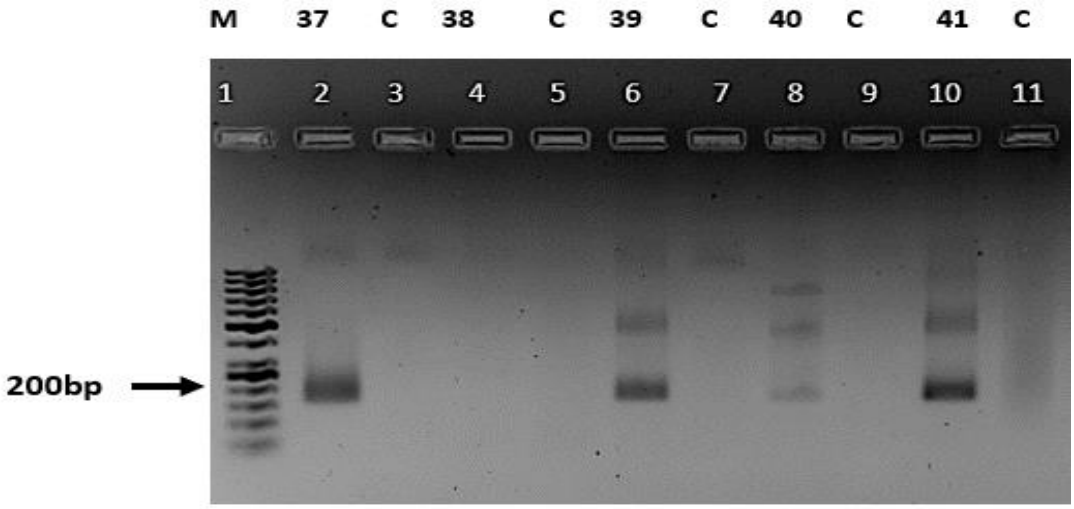

Figure 3-2: RPA generated phospholipase C (*plc*) amplicons using temperature titritration. Lane 1: MW marker (GeneRuler low range ladder), Lanes 2-11: PCR done at indicated temperature, Lanes 2,4,6,8,10: contained input DNA of approximately 23 ng/PCR, Lanes 3,5,7,9: contained PCR with water (negative control PCRs).

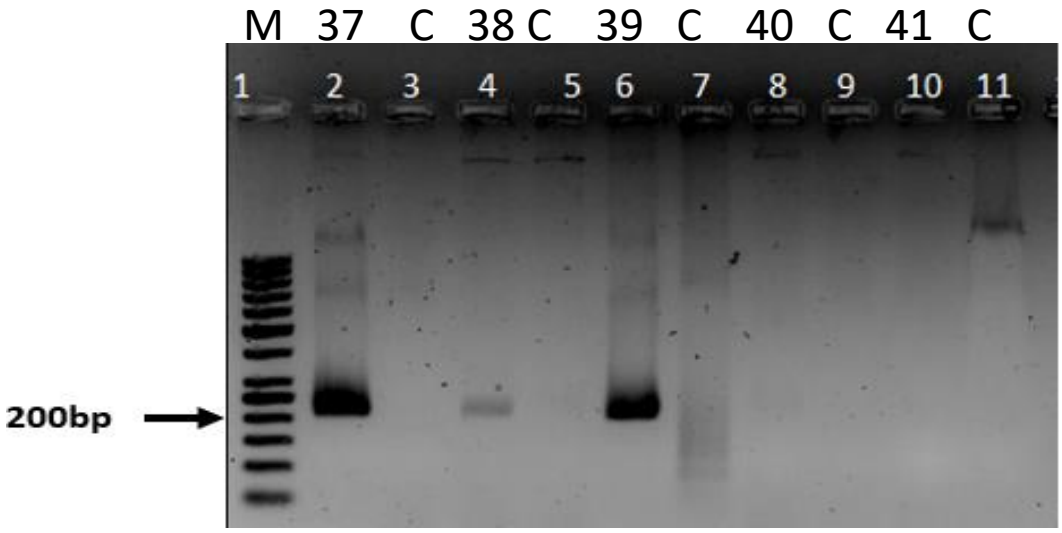

Figure 3-3: RPA of the *E. coli* 0157:H7 gene. Temperature titration was done. Lane 1: MW marker (Generuler low range ladder), Lanes 2-11, PCR done at indicated temperature; Lanes 2,4,6,8,10: contained input DNA of approximately 23 ng/PCR, Lanes 3,5,7,9, 11: contained PCR with water = negative control PCRs. The expected band size is 216 bp, as described in previous work.

RPA for *L. pneumophila* was set up as reported previously (*40*), also generating the expected amplicon of correct size (as previously reported (165 bp). However, we observed some smearing for the negative control PCR. Thus, the primers were checked, via conventional PCR, to discern if this primer set generate non-specific amplification via conventional PCR (DNA separated by heat and not with an enzyme as done in RPA assays). The conventional PCR did not generate non-specific amplification, but primer dimers can be seen in the negative control (Figure 3-4). The RPA potentially could be optimised with a primer titration, if the primer dimer formation is a problem at higher temperature (as in the conventional PCR), it could explain the smearing in the RPA (where a much lower temperature is used and thus one expect more non-specificity or primer dimer formation. Nevertheless, the RPA for *mip* generated the correct band, and clearly more amplicons were generated via RPA than in the conventional PCR.

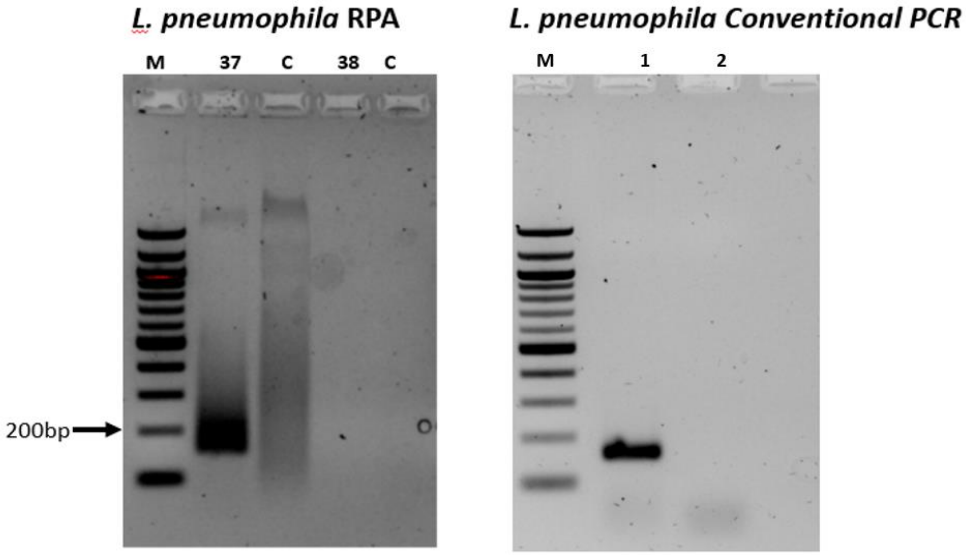

Figure 3-4: Isothermal and conventional PCR for *L. pneumophila mip* gene. On the left is gel with amplicons generated via RPA, on the right via conventional PCR. Lane 1: PCR with template, Lane 2: PCR negative control, with water. Lanes 37, 38: indicate the temperature used for RPA amplification, Lanes marked as C: negative control with water.

In summary, recombinase polymerase amplification of DNA extracted from the 3 pathogens was successfully demonstrated. However, some further optimisations potentially could be done, to minimise non-specific amplification. The amplicons shown above were not cleaned up (as is done in many published RPA papers), so that any non-specific smearing could be seen (if present) and is useful to inform if further clean-up and optimisation is needed. Nevertheless, the impact of these amplicons on detection via lateral flow assays might be minimal (if too little and not labelled properly, it might not be detected at all, but this needs to be tested).

3.3.4 Detection of dual labelled amplicons

The next step was to discern if the lateral flow device (PCRD, Abingdon Health, UK) would detect amplicons labelled with DIG and biotin (*C. perfringens* and *L. pneumophila*) or 5-FAM and biotin (*E. coli* 0157:H7) could be detected on lateral flow strips. The minimum amount of DNA to use in a PCR that could result in visible amplicons was also determined. For this, both conventional PCR and RPA was done, and amplicon generation verified via agarose gel electrophoreses and lateral flow strips. However, for this step RPA PCR was done using the lyophilised TwistAmp Basic kit; this kit contains the same components as the Liquid Basic kit, but all reagents (except the primers, DNA template and Magnesium Acetate) must be added after the lyophilised powder is rehydrated. This kit also comes in handy 8-tube strips and is easier to handle than the Liquid Basic kit whose liquid components are highly viscous, and pipetting could easily be less accurate due to the viscosity (see Figure 3-5 for picture of one strip). This kit also would be a better option as kit reagents are lyophilised, while the Liquid Basic kit comes in bulk and needs to aliquoted into individual vials to prevent repeated freeze/thaw cycles (could impact PCR efficiency).

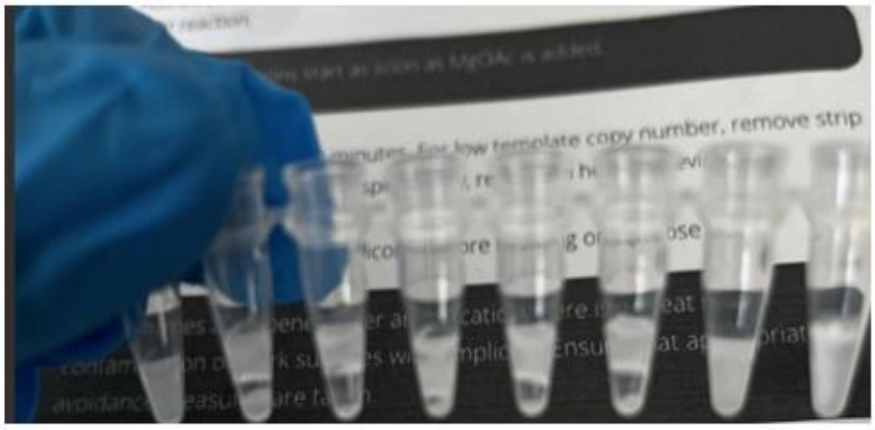

Figure 3-4: A test strip from the TwistAmp Basic kit, containing 8 PCR tubes with lyophilised RPA reagents. A rehydration buffer (provided in the kit) is added, plus the primers, water, template DNA, and magnesium acetate; the latter is added last, as it initiates the PCR reaction, which starts immediately.

To **discern the minimum amount of input DNA (LOD)** needed for detectable amplicons, a stock concentration of 10 ng/µl of DNA was prepared. This was used to prepare serial dilutions from 1 ng of DNA to 10^{-6} ng of DNA. As per the protocol, 1ul of DNA was added per PCR reaction. Both conventional and RPA amplification was done, as both have utility, the conventional PCR could be done in laboratories where infrastructure is available. To capture amplicons on the lateral flow device, the labelled primers were used for amplification.

Amplicons were detectable in both agarose gels and the lateral flow strips when conventional PCR was used (Figures 3-6 to 3-10). For *L. pneumophila*, labelled amplicons generated with conventional PCR was clearly visible when as little as 0.01 ng of DNA (0.01 n- or 10 picogram (pg)) of template DNA was used. On the agarose gel, a very faint band was detected even at 1 pg of input template (Lane 6). However, for this, one could overexpose the gel and it should be visible more clearly (Figure 3-6). Aliquots from the same amplicon mix were mixed with the PCRD buffer (provided in the kit) and loaded on the strips.

As seen in Figure 3-7, the lateral flow strip detected amplicons when as little as 1x 10^{-3} ng of template was used in the conventional PCR. In other words, 1 pg of input template enabled generation of detectable amplicons. This is tenfold more sensitive than detected with agarose gel electrophoreses. However: to verify this result and ensure that this specific amount can be detected with confidence and is reproducible, one has to do enough replicates, with real world samples, to enable statistical testing. Since water samples differ in microbial load, chemical composition, and bioburden of various origin, it would be best to test diagnostic parameters such as repeatability, reproducibility, and sensitivity for different water sample types, to verify that the diagnostic parameters are similar or different/ unique for different water sample types. It should be noted that a good DNA extraction method/kit that will produce pure DNA, free of inhibitors, etc. irrespective of the water origin, potentially could enable a similar LOD irrespective of sample origin. Yet this has to be verified (and not assumed). Also noted is that unlabelled primers or primer dimers do not result in false positive results as no amplicons are visible below the 1 pg input sample (i.e. the strip marked as Lpn -5). Furthermore, when comparing agarose vs LFD detection, the sample with DNA template input of 1 pg yielded amplicons that had better visibility on the lateral flow strip, while it was very faint when visualised with UV transillumination of amplicons in the agarose gels.

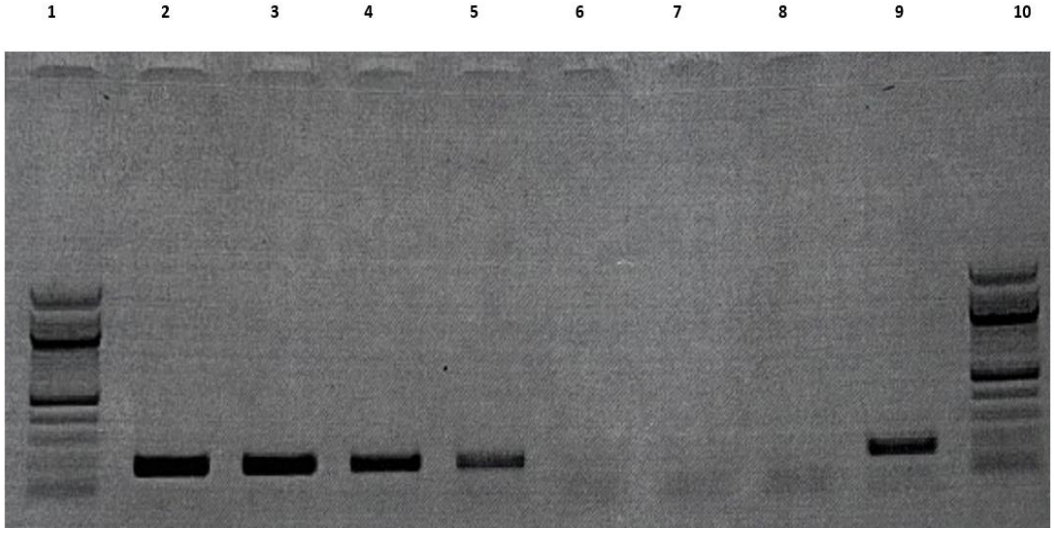

Figure 3-5: Conventional PCR of DNA from L. pneumophila. Amplicons were prepared from serial dilutions of DNA, with input range from 10 ng to 1x 10-7 ng of DNA. Lanes 1 and 10: molecular weight marker, 100bp Quickload DNA ladder; Lane 2: 10 ng input DNA, Lane 3: 1 ng input DNA; Lane 4: 0.1 ng input DNA, Lane 5: 0.01 ng input DNA; Lane 6: 1 pg input DNA; Lane 7: 0.1 pg input DNA; Lane 8: negative control with water as template; Lane 9: positive control, DNA extract of *L.pneumophila* previously used in PCR and confirmed to generate correct amplicon.

Figure 3-6: Lateral flow detection of L. pneumophila. Amplicons are labelled on both ends, on 5' end with DIG and on 3' end with Biotin. Only the amplicons generated with input DNA of 10 ng (Lane 2 on gel, -3 on the strip) as well as in Lane 5-7 in Figure 3-1 were used for LFA detection (Lane 5: 0.01 ng aka 10 pg input DNA; Lane 6: 1 pg input DNA; Lane 7: 0.1 pg input DNA). C. LFD control line, should always be positive, indicating that the strips are functioning correctly. 10fold serial dilutions were prepared from 10 n/ul of DNA, thus: Lpn -3 = input DNA in PCR was 10 ng x 10-3 dilution or 0.01 ng DNA or same as 10 picogram input DNA.

For comparison of labelled *C. perfringens plc* amplicons (Figures 3-8 and 3-9), input template of 1x 10^{-3} ng DNA also yielded visible amplicons on agarose gels and the LFD device. As for *L. pneumophila,* input DNA of 10 ng and 1 ng yields amplicons that are clearly visible. Interestingly, the positive control was faint, potentially as this DNA was from an older extraction, and had been freeze/thawed multiple times to use for positive controls previously. Also, the dual labelled *plc* amplicons did not provide strong signals like the *L. pneumophila* amplicons, suggesting that potentially less amplicons are made for this *C. perfringens* PCR and thus implies more optimisation needed. Nevertheless, it was demonstrated that labelled amplicons are detectable using a commercial LFD device. Lastly, we compared agarose gel and lateral flow detection of the *E. coli* 0157:H7 *rfbE* gene target, also using conventional PCR and labelled primers. This primer set was labelled with biotin

and 5-FAM respectively, so should be detected at test Line 2. As seen in Figures 3-9 and 3-10, visible amplicons are detected clearly with a minimum template input of 10 pg of DNA, via on agarose gels and on the lateral flow device. For this microbe, 1 pg of template DNA yielded amplicons that was more visible compared to the other two organisms tested in this study.

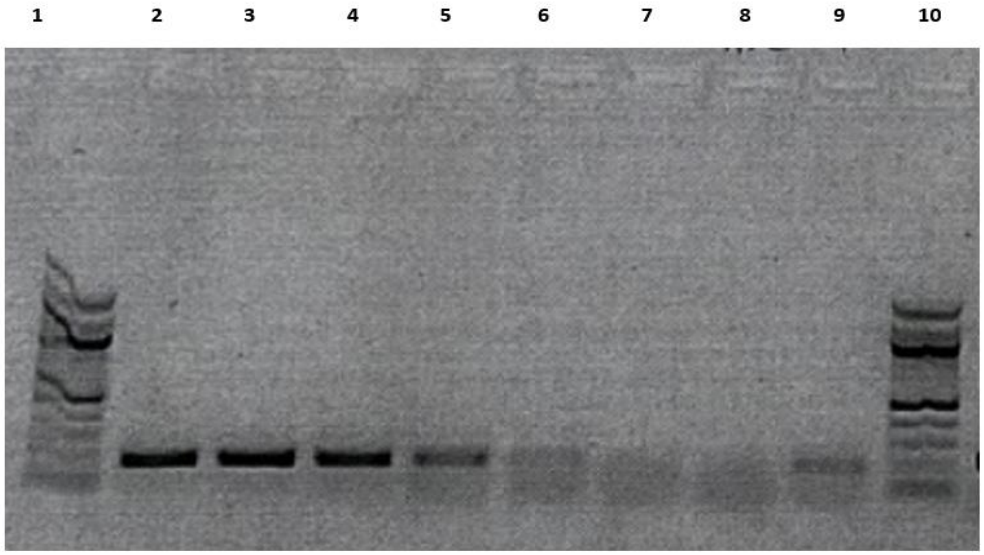

Figure 3-7: Conventional PCR of DNA from *C. perfringens*. Amplicons were prepared from serial dilutions of DNA, with input range from 10 ng to 1x 10^{-7} ng of DNA. Lanes 1 and 10: molecular weight marker, 100bp Quickload DNA ladder; Lane 2: 10 ng input DNA, Lane 3: 1 ng input DNA; Lane 4: 0.1 ng input DNA, Lane 5: 0.01 ng input DNA; Lane 6: 1 pg input DNA; Lane 7: 0.1 pg input DNA; Lane 8: negative control with water as template; Lane 9: positive control, 30 ng of DNA of *C. perfringens*. NOTE: The positive control did not amplify well.

Figure 3-8: Lateral flow detection of *C. perfringens* amplicons generated by conventional PCR. Amplicons are labelled on both ends, on 5' end with DIG and on 3' end with Biotin. Only the amplicons generated with input DNA of Lane 2 (10 ng input DNA) as well as in Lane 5-7 in Figure 3-1 were used for LFA detection (Gel Lane 5: 0.01 ng aka 10 pg input DNA; Lane 6: 1 pg input DNA; Lane 7: 0.1 pg input DNA). 10fold serial dilutions was prepared from 10 n/ul of DNA, thus: plc $^{-3}$ = input DNA in PCR was 10 ng x 10-3 dilution/0.1 ng DNA/10 picogram input DNA.

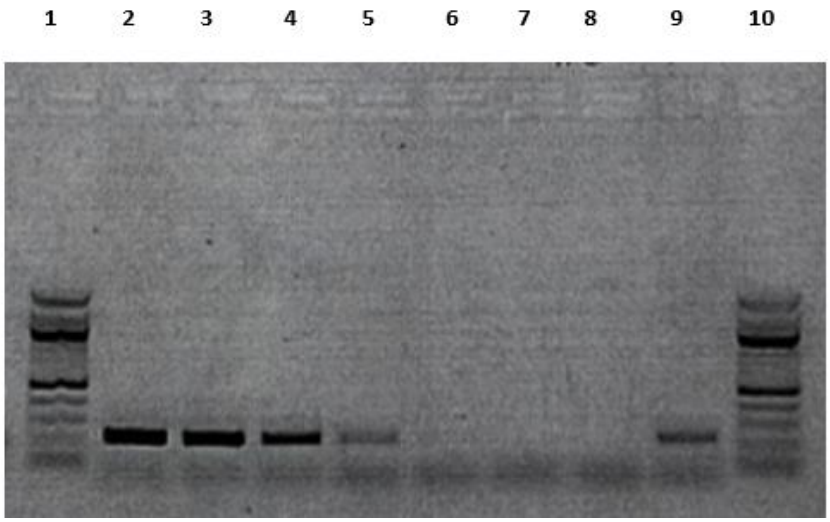

Figure 3-9: Conventional PCR of DNA from *E. coli* 0157:H7. Amplicons were prepared from serial dilutions of DNA, with input range from 10 ng to 1x 10^{-7} ng of DNA. Lanes 1 and 10: molecular weight marker, 100bp Quickload DNA ladder; Lane 2: 10 ng input DNA, Lane 3: 1 ng input DNA; Lane 4: 0.1 ng input DNA, Lane 5: 0.01 ng input DNA; Lane 6: 1 pg input DNA; Lane 7: 0.1 pg input DNA; Lane 8: negative control with water as template; Lane 9: positive control, 30 ng of DNA of E. coli *0157:H7.*

Figure 3-10: Lateral flow detection of E. coli 0157:H7 amplicons generated by conventional PCR. Amplicons are labelled on both ends, on 5' end with DIG and on 3' end with Biotin. Only the amplicons generated with input DNA of 10 ng (Lane 2) as in Lane 5-7 in Figure 2-5 were used for LFA detection (Lane 5: 0.01 ng aka 10 pg input DNA; Lane 6: 1 pg input DNA; Lane 7: 0.1 pg input DNA). 10fold serial dilutions was prepared from 10 n/ul of DNA, thus: 0157 -3 = input DNA in PCR was 10 ng x 10-3 dilution/0.01 ng DNA/10 picogram input DNA.

To summarise, when dual labelled amplicons are generated via conventional PCR, input template DNA as little as 10 ng generates enough amplicons to be detected via both agarose gel electrophoreses (and UV transillumination) as well as the lateral flow device. The data above also demonstrated the utility of the specific lateral flow device used in this study, namely the PCRD cassette. As dual labelled amplicons were successfully detected via a lateral flow device, the next step was to confirm the actual aim of this study, namely, to use RPA instead of conventional PCR and do detection via the lateral flow device. Therefore, RPA reactions was setup, using the TwistDx Basic kit and labelled primers, using 37°C as the amplification temperature and the reagent concentrations as per the kit manual.

3.3.5 Lateral flow detection from dual labelled amplicons generated via RPA

The PCR device was tested first (to see if it works) using only one concentration of DNA shown earlier to always yield positive PCR amplicons, i.e. 10 ng input DNA. Secondly, it was important to establish that non-specific amplification observed on agarose gels does not generate false positive detection on the LFD device. As seen in Figure 3-12, all three microbes yielded positive results, although the *C. perfringens plc* was only faintly positive. This was a concern, and new DNA was extracted from fresh cultures, before further testing was done.

Figure 3-11. First test to discern if labelled amplicons are detected on PCRD device. Lpn = *L. pneumophila*; Cp = *C. perfringens*; 0157 = *E. coli 0157:H7*. 10 ng input DNA and 37°C used as amplification temperature. Test line 1 must detect *L. pneumophila* and *C. perfringens*, while test line 2 detects *E. coli* 0157:H7.

New serial dilutions of DNA were prepared, same range as previously described, to do DNA titration for RPA. ***For LFD detection:*** 84ul of diluent from kit was mixed with 6ul of PCR amplicons. As per instructions, 75 µl of this was loaded onto the strips and results read within 10minutes (and not thereafter, as instructed). RPA PCR, unlike conventional PCR yield faint bands on LFD strips, when input DNA of 10 pg were used. Lower amount of DNA (1 pg and lower) yielded faintly detectable results; these were not visible on the agarose gels (gels not shown here, but resembles *C. perfringens* smearing in Figure 3-13, thus suggest that an input of DNA lower than 10 pg cannot be reliable detected). Furthermore extensive (and appropriate) validation of the lower limit of detection is necessary, using multiple replicates of different real-world samples to discern the confidence with which lower than 10 pg of DNA will be detected.

3.4 SUMMARY

Proof of principle is provided that dual labelled PCR amplicons of pathogens, when labelled on end with biotin (to enable streptavidin-based detection) and either DIG OR 5-FAM on the other end can be detected using a commercially available lateral flow device. PCRs (especially for *C. perfringens*) should be optimised to avoid non-specific smearing, typically associated with isothermal PCR. Our results thus far show that non-specific amplification occurs if amount of DNA in PCR reaction is 1 pg or lower, possibly the reason for faint detection of bands on the LFD strips. This indicates a risk for false positive results, so fine-tuning the PCR must be done, and validation of the lower limit of detection using multiple replicates. Suggestion is that validation is done as prescribed for real-time PCR validation, where at least 20 replicates are run to test the amplification and detection of a specific input amount of DNA, typically the lowest amount detected, as well as one concentration above and below this perceived limit of detection. This enables statistical calculation of the probability to detect true positives vs false positives.

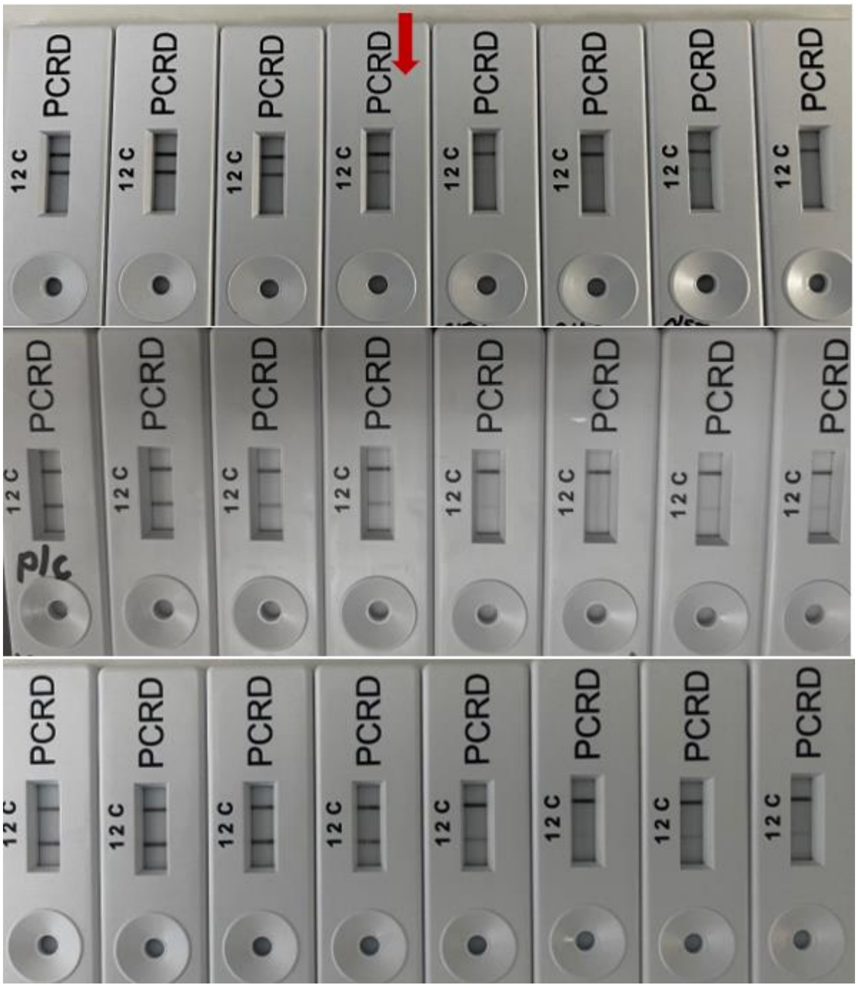

Figure 3-12: Lateral flow detection of *E. coli 0157:H7*, *C. perfringens* and *L. pneumophila.* Order top to bottom) amplicons generated by RPA PCR. From left to right DNA input in PCR was 10 ng, 1 ng, 0.1 ng, 10 pg, 1 pg, 0.1 pg, 0.01 pg. The last device on right= PCR with no input DNA, water as template =negative control. The red arrow suggests the potential lowest amount of input DNA template (10 pg) that will yield a reliable, reproducible positive PCR. This must be validated doing replicates of PCR of this input DNA.

CHAPTER 4: VALIDATION OF METHOD USING ENVIRONMENTAL WATER SAMPLES

4.1 INTRODUCTION

Different water sample types represent varied challenges for DNA extraction and downstream molecular testing. Low abundance or overabundance of microbes in a sample both could result in incorrect results if samples not appropriately processed. Furthermore, the presence of various chemicals in water also could interfere with amplification of nucleic acids, as demonstrated by other researchers such as Lemarchand and co-workers (*44*). These factors will be considered in this project, to ensure appropriate processing of samples.

Samples with low microbial abundance might have too little of a specific microbial target, leading to potential false negative result of a test. To counter this, samples are concentrated on filters, by processing large volumes of water through filters. Various devices are on the market, including handheld syringes and manual filtering of water, or larger beaker devices with embedded filters where water are filtered with the aid of vacuum pumps. Filters will be rinsed off in smaller volumes of buffer or growth media or could be cut into smaller sections and stored until further processing. Samples with high microbial burdens could influence the filtering process, and even impede nucleic acid extraction. In this case, filters used to concentrate material might become blocked. DNA extractions using commercial, column-based filters will have similar problem. A solution is to optimise the amount of water to filter, and adapt volumes used for DNA extractions appropriately.

Samples containing chemicals further impacts preparation of DNA. For example, residual chlorine in treated wastewater should be neutralised/removed/deactivated as chlorine solutions degrade nucleic acids. Specific DNA extraction kits are on the market for extraction of DNA from water (e.g. the Ultra Clean Water DNA isolation Kit (Mo Bio), but it is best to use commercial kits available on regular supply. Hence an investigation of kits available locally, with no delay in delivery and at cost-effective prices, is advisable. This includes investigation of methods to avoid DNA extractions requiring centrifuges, fume hoods and chemical waste disposal systems. Extraction free methods, while not freely used yet, is best for work done in the field and at point of care. It also minimises risk for technical mistakes (e.g. switching of samples, cross contamination, etc.); ideally a method where the sample are directly used for PCR is ideal, but using different water sample types will require optimisation for the different types. Unfortunately, unless filtered material can be treated to remove PCR inhibitors before using “extraction free” methods, this method would potentially not be optimalfit for purpose (yet this should be confirmed in studies).

In this part of the study, the utility of recombinase polymerase amplification to amplify and detect pathogen DNA extracted from real world water samples would be analysed. Water samples from various environmental origins were provided by the Scientific Services laboratory (City of Cape Town). Due to the nature of different chemical inhibitors that might be present, as well as different burdens of solid and microbial matter in the various water types, we need to verify methods to filter and process different water types. Due to time limitations, this aspect was not explored in this study. Instead, provided water samples were filtered and DNA extracted using a filter column-based DNA extraction method; this kit contained an additional step to remove PCR inhibitors.

4.2 WATER COLLECTION AND PROCESSING

4.2.1 Water collection- sampling points

Scientific Services (City of Cape Town) provided water samples, collected during routine sampling across various sites serviced by the relevant department. Samples and characteristics are reported in Figure 4-1.

SAMPLE	APPEARANCE	How much filtered?
SWIMMING POOL	CLEAR	2 litres
TIDAL POOL	CLEAR	1 litre
Standpipes, water tanker	CLEAR	2 litres
RIVER	SLIGHTLY MURKY	200 ml
POTENTIAL REUSE H_20	CLEAR, SOME SPECKLES OF SAND?	1 litre
WASTEWATER	VERY DIRTY, HIGH BIOBURDEN	50 ml
INDUSTRIAL WASTE	VERY DIRTY, HIGH BIOBURDEN	2x 50ml
TAP WATER	RECENT BURST PIPE IN BUILDING, YELLOW	100ml

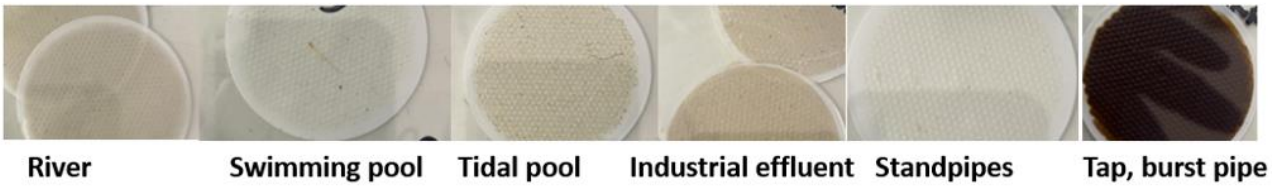

Figure 4-1: Water samples and characteristics. The appearance of water before filtration through 0.45 µm filters are described. The tap sample is not from City of Cape Town but was taken one day after repairs of a burst pipe in an unidentified building with a recent pipe burst. As expected, this water would be discoloured and potentially would contain biofilm material; the latter is known for biofouling water pipes. Filters are shown to visualise the different bioburden between the samples.

4.2.2 Sample pre-treatment

As expected, due to differences in potential bioburden (both eukaryotic and prokaryotic material), different volumes of water were filtered before filters got clogged. This is an important factor to consider when doing molecular testing, bearing in mind that very low numbers of a bacterial target present in a sample, will be negatively impacted. Unlike culture based methods, if low numbers of a target bacterium is present amongst an excessive number of other microbes, the PCR might be negatively impacted and yield a false negative result. This aspect should be studied further and should be done with different water sample types (representing potentially different matrices and PCR inhibitory compounds). DNA extraction Kits containing inhibitor removing steps should be prioritised for validation with molecular detection methods.

4.2.3 DNA extraction and RPA amplification

For the samples reported above, DNA extractions were done using the Faecal/Soil Microbe Miniprep kit (https://files.zymoresearch.com/protocols/_d6010_quick-dna_fecalsoil_microbe_miniprep_kit.pdf, Zymo Research), chosen as it contains a mechanical lysis step, it is optimised to extract DNA from faecal matter (thus potentially ideal for human sewage) and it contains a step where potential PCR inhibitors are removed from extracted DNA. At the time this report was finalised, no peer reviewed papers yet have reported on this

kit and its utility to extract DNA fit for purpose from environmental water samples (although the kit indicates water samples could be extracted. Noteworthy though, one study reported this kit as best performing extraction kit to produce reliable, reproducible data for extraction of DNA from *Cryptosporidium* from faecal matter (*45*). As this pathogen is a known resilient pathogen that could escape chemical treatment of sewage, it should be investigated further with regards to water from diverse environments.

Before RPA testing was done, the extracted DNA was tested first to check if all the DNA is amplifiable. For this, conventional PCR was done with 16s rRNA primers (Table 3-1), to confirm the presence of bacterial DNA and that extracted DNA is fit for purpose.

4.3 RESULTS AND SUMMARY

DNA extractions from the filtered material yielded variable concentration of extracted DNA, ranging from less than 10 ng/µl to very high (>300 ng/µl); this of course was related to the bioburden. All samples amplified the relevant gene, with an amplicon size of approximately 1.5kb (Figure 4-2). Significant non-specific amplification was also observed, not an unusual observation when DNA contains a mixture of bacterial and eukaryotic DNA as one of the primers is known for off target binding.

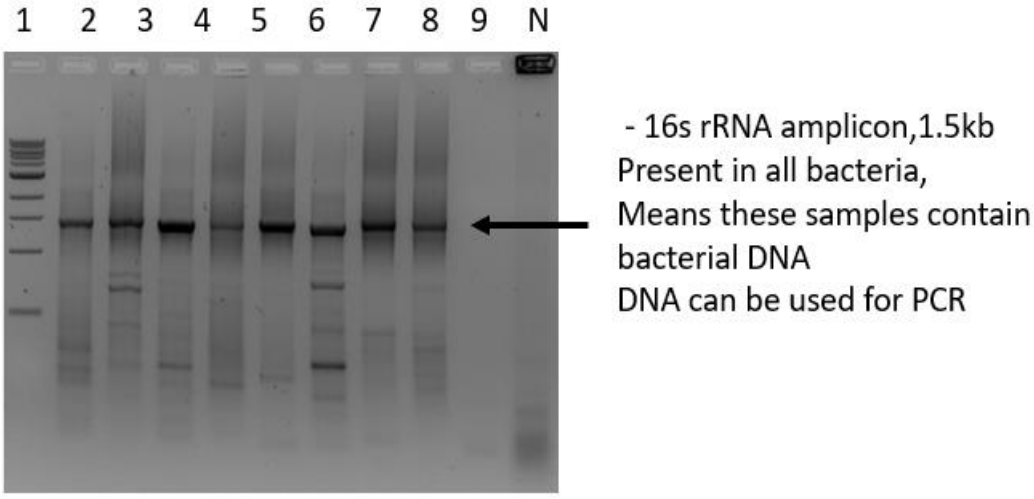

Figure 4-2: Confirmation of the presence of bacterial DNA in DNA extracted from water samples. Lane 1: MW marker, 1kb Quikload ladder, NEB, Lanes 2-8: DNA from water from a swimming pool, a tidal pool, a standpipe (used for water tankers), a river, post-treatment water for potential re-use, treated wastewater, industrial wastewater, unnamed tapwater from a recently repaired burst pipe. Lane 9: negative control, using molecular grade water, N, DNA from *C. perfringens* colony – not pre-treated or extracted.

As abovementioned PCR confirmed that bacterial DNA was present in the water samples and that it is fit for purpose (i.e. amplifiable), RPA PCR was done with DNA from the tidal pool, the post-treatment sample, the industrial effluent as well as the unnamed tap water. Note that it is expected that the latter potentially would contain *L. pneumophila* DNA, as building water infrastructure is a known host of this organism. As seen in Figure 4-3, pathogen DNA amplified and was successfully detected via the lateral flow device. *E. coli* 0157:H7 was detected in DNA from the post-treatment sample and the industrial effluent sample, while *L. pneumophila* SG1 was detected in all water sample types, including the water from a recently repaired pipe burst. This is not unexpected, as this organism lives in environmental waters and is a known resident of building water pipes. Lastly, as expected, *C. perfringens* was detected in the post-treatment and industrial effluent sample, but not in the tidal pool sample.

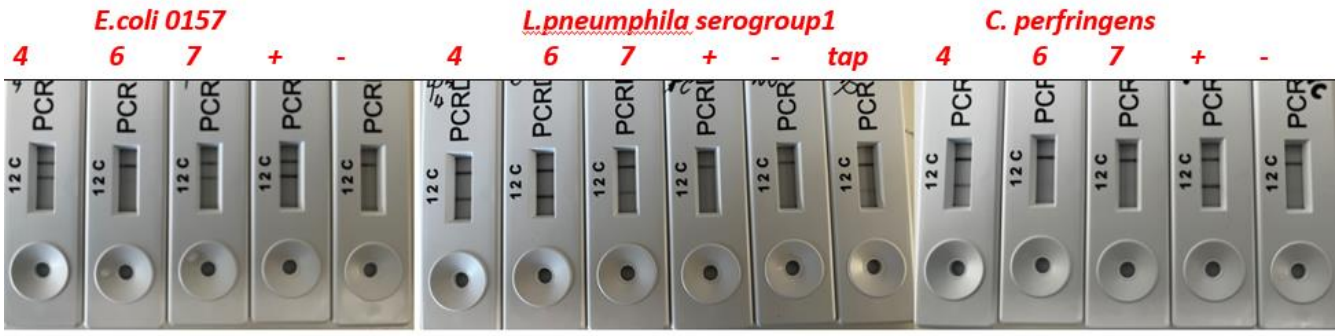

Figure 4-3: Detection of pathogen DNA in water samples via RPA and LFA. 4: post-treatment water for possible reuse; 6: tidal pool; 7: industrial effluent; +, positive control; -, negative control (molecular grade water).

It is important to mention, at this point, that the DNA amplification method reported here only report on the presence of DNA, **not viability of organisms**. For that, samples must be cultured, or a viability PCR must be done to detect only DNA from viable organisms and not residual DNA remaining from dead organisms.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Lateral flow detection of dual labelled PCR amplicons was successfully shown for all 3 pathogens. The procedure was optimised using ideal conditions, that is purified DNA from cultured bacteria was used for target amplification. It is easy to set up, especially using the lyophilised kit TwistAmp Basic kit used in this study. A drawback is that only one RPA kit is available globally, manufactured by only once company. Potentially it should be explored to develop in house RPA assays as described originally(*47*).

Lateral flow detection via the commercial PCRD device was successful with amplicons generated via classical PCR (where DNA separated by heat during PCR) and via recombinase polymerase amplification (no need for a thermal cycler, done at a single temperature). Again, the procedure was simple and amplicons visible within 5minutes of exposure to the strip.

The study showed that if about 10 pg of the targeted DNA is present in a PCR, it will generate sufficient amplicons to be detected clearly on both agarose gels and a commercially available lateral flow device. This was true for all 3 microbes tested. However, this validation was done using purified DNA, as the potential of the RPA method was evaluated and confirmed that it is fit to evaluate with titrations of bacterial cells (minimum 1 target bacterial cell per filtered water sample). A future, extensive validation should be done, using multiple replicates (sufficient for appropriate statistical analysis) of DNA and with DIFFERENT water types, with DNA extracted via a kit suitable for the different water sample types.

It can be concluded that further optimisation is done to minimise the faint positive (potentially false positive) bands observed when RPA PCR DNA input is 1 picogram and below. This phenomenon might be related to the fact that isothermal methods generate a lot more PCR products compared to conventional PCR. Therefore, if a laboratory chose isothermal PCR as a method, some optimisation is advised to validate the method in house. Also, each laboratory must ensure that the PCR conditions as described in a paper, results in the same output if done in own facility and with the specific DNA extraction methods used in the specific facility. The isothermal PCR for *C. perfringens* need more optimisation that those for *L. pneumophila* and *E. coli* as more non-specific smearing are observed in the clostridial isothermal PCR with 1 pg and less input DNA, both in gels and on the lateral flow device. An optimised assay, with minimal primer dimers or non-specific smearing is ideal to prevent false positive results.

Using pure bacterial cultures, sufficient DNA was extracted from a minimum of 10^8 cells and detectable using laboratory instrument. However, to be applicable, real world water samples should be spiked with cell numbers ranging from 1-10 000 cells, extractions done, and the presence of the target microbes detected via PCR. Due to time constraints, this was not done to date; will be a future investigation for one of the students on this study. DNA was successfully extracted from all provided water samples using a kit promoted for extraction from faecal matter and soil, though no peer reviewed publications could be found where this kit was used for extraction from water. The DNA was found fit for purpose and PCR amplicons were generated successfully using both conventional PCR and RPA. This kit is locally available, very easy to use and well-priced. We concluded that this kit could potentially be useful for extractions from water, notably as it is reasonably priced, available as a column based and a magnetic bead extraction method. It also had been evaluated in the literature and shown to extract DNA from Cryptosporidium, an organism known to survive water treatment, and which is needed to be monitored when reclaimed water is to be used successfully and reliably.

Cost comparison between culture-based methods and molecular detection, as initially planned, is not reported here. With the current financial climate, prices of reagents spiked within the last 3 years, so a fresh evaluation is necessary. Prices obtained for extraction kits and other reagents a year ago (for cost comparison) is not applicable any longer and a broad comparison would be more practical (not using precise costs, but a range (e.g., grouped within a specific bracket, e.g. 5-10K instead of specifically stated quotes.

Lastly, but important, although the RPA method described in this report was successfully demonstrated, it is limited in use currently as it does not provide evidence for viability of microbes. Thus, future work should include testing this technique in the presence of PMA plus viable and non-viable organisms, to develop a viability PCR. This will be more valuable and useful for point of care testing.

5.2 RECOMMENDATIONS

RPA amplification of bacterial DNA and lateral flow detection of generated amplicons, labelled with relevant molecules to be captured on the specific lateral device used, is a viable option to explore further for point of care work.

Future work should explore **inclusion of an internal amplification control**, relevant for diagnostic confidence in the test. For the specific device used in this study (PCRD), two test lines are available, meaning that one could develop the method further to include the detection of an internal amplification control (IAC) added before DNA extraction and extracted with the relevant sample. Amplification of this IAC would confirm that that amplifiable DNA extracted, failure to amplify would indicate that either extraction was not successful or that amplification is inhibited by the presence of PCR inhibitors. In this regard, it is could be ideal to use the 16s ribosomal RNA gene, as it is present in virtually all bacteria, most often is multicopy, in other words more sensitive to detect. However, the primer set to be used should be optimised and in such a way that amplification of this gene does not influence the LOD of the actual bacterial target being screened for. Other diagnostic controls are also available commercially and could be evaluated.

The PCRD device is provided in individually sealed packets, stored at room temperature and is packaged in a cassette, thus one does not need to put a lateral flow strip in a tube and add buffer to it. This device also is cost-effective (150 devices were purchased for approximately R15 000, thus R100 per imported strip). Cost comparisons should be done with local biotechnology companies who manufacture similar lateral flow strips and potentially comparisons could be done for reliability, costs, etc.

For this study, the 3 DNA extraction kits tested were not evaluated using real world samples, instead we aimed to discern the minimal amount of bacterial cells (counts verified using serial dilution and plating) needed to yield DNA that is detectable with laboratory instruments. This was done to verify that the specific kits actually effectively generate DNA fit for purpose. These kits now have to be evaluated with real world samples; where samples are proven negative for the specific pathogens, it should be spiked with 1-10 000 cells to discern if target bacterial DNA will be detected amongst a complex collect of various organisms. While the (fairly new) Faecal/Soil Zymo kit, were used to extract DNA from supplied real world water samples and the RPA methods employed on this DNA, time was not sufficient to do a proper laboratory validation with real world samples. This should be followed up in future work, indeed is planned for one of the students in the study. This last kit also is available in the form of a magnetic bead extraction kit; the latter is highly useful for point of care extractions. It is recommended therefore that this specific kit range should be explored as other studies have found it reliably and reproducibly generate DNA from faecal organism. Thus, potentially valuable for sewage samples and faecal contaminated water.

The effect of room temperature storage on the efficiency of the Twistamp Basic lyophilised material was not tested in this study. This is an important aspect for point of care work and storage in places without cold chain storage. This had been explored by one study only thus far, and it was found that storage at a temperature of

45^0C renders the kit less effective to amplify HIV nucleic acid after 3 weeks(*48*). This is a limitation to be explored in future studies.

Lastly, the RPA method is reported to work also using human body temperature (*6*), or if placed inside a pouch which then is placed in close contact with the human body. Body heat was shown to generate results 100% correlated with RPAs done using a heated device. These research reports had not been explored or verified by other studies but should be explored as the use of body heat would exclude even the need for a heated device in remote setting. The only limiting step then would be DNA extractions where at least a centrifuge is needed.

5.3 HUMAN CAPITAL DEVELOPMENT AND OTHER OUTPUTS

Two Master of Science students participates in this project. Both are and were exposed to new techniques and expanded their skills sets. One of these students currently is writing his thesis. The second student, also an employee of the local municipality and involved in water quality management already, has decided to upgrade as the project could lead to a PhD degree, with the exploration of a viability RPA method, optimisation of DNA extractions using different kits not yet validated for water extractions. The screening of local water samples also afford opportunity to explore if the presence of *C. perfringens* in local water environments indeed could reliably be correlated with the presence of *Cryptosporidium* and other resilient organisms. A project proposal had been prepared already and is currently being evaluated by the main supervisor, followed by relevant upgrade application.

Peer reviewed outputs.
One **review** had been prepared for publication. This deals with isothermal PCR methods and detection of pathogens in water. Will submit for publication within 2 months following this final report.
A **second paper**, describing the development of *C. perfringens* had been discussed. Some extra work needs to be done before there is sufficient data for publication. This entails optimisation to remove observed background RPA primer dimers, limit of detection studies with real world samples and further exploration of DNA extracted from spores and amplified via RPA. Not reported in this final research report, but briefly explored by one student, was the use of a so-called extraction free kit to extract DNA from *C. perfringens*. This type of kit might not be practical if excessive biomaterial and chemical PCR inhibitors are present in a sample but could have potential utility for reclaimed and repurposed water, where little bioburden is expected and thus could be filtered in larger volumes, filters swabbed and rinsed off in minimal buffer (water or PBS or TE), then lysed as per the specific kit instruction. This is ideal as the method uses a heating block for incubation of a sample in the presence of cell wall degrading enzymes, before the sample is boiled and PCR ensued.
Other potential papers: A combined paper reporting on viability RPA for the 3 pathogens – ideal, but would require extensive work to be done still.

Other output: One MSc and potentially one PhD thesis.

REFERENCES

1. M. V. Yates, Classical Indicators in the 21st Century—Far and Beyond the Coliform. *Water Environment Research* **79**, 279-286 (2007).
2. D. W. Griffin, E. K. Lipp, M. R. Mclaughlin, J. B. Rose, Marine Recreation and Public Health Microbiology: Quest for the Ideal Indicator: This article addresses the historic, recent, and future directions in microbiological water quality indicator research. *BioScience* **51**, 817-825 (2001).
3. H. D. de Paz, P. Brotons, C. Muñoz-Almagro, Molecular isothermal techniques for combating infectious diseases: towards low-cost point-of-care diagnostics. *Expert Rev Mol Diagn* **14**, 827-843 (2014).
4. P. Maffert, S. Reverchon, W. Nasser, C. Rozand, H. Abaibou, New nucleic acid testing devices to diagnose infectious diseases in resource-limited settings. *Eur J Clin Microbiol Infect Dis* **36**, 1717-1731 (2017).
5. K. T. L. Trinh, N. Y. Lee, Fabrication of Wearable PDMS Device for Rapid Detection of Nucleic Acids via Recombinase Polymerase Amplification Operated by Human Body Heat. *Biosensors (Basel)* **12**, (2022).
6. Z. A. Crannell, B. Rohrman, R. Richards-Kortum, Equipment-free incubation of recombinase polymerase amplification reactions using body heat. *PLoS One* **9**, e112146 (2014).
7. I. T. Hull *et al.*, Isothermal Amplification with a Target-Mimicking Internal Control and Quantitative Lateral Flow Readout for Rapid HIV Viral Load Testing in Low-Resource Settings. *Analytical Chemistry* **94**, 1011-1021 (2022).
8. D. Rios-Covian *et al.*, Different metabolic features of Bacteroides fragilis growing in the presence of glucose and exopolysaccharides of bifidobacteria. *Frontiers in Microbiology* **6**, (2015).
9. I. Dieng *et al.*, Mobile Laboratory Reveals the Circulation of Dengue Virus Serotype I of Asian Origin in Medina Gounass (Guediawaye), Senegal. *Diagnostics (Basel)* **10**, (2020).
10. A. Nocker, C. Y. Cheung, A. K. Camper, Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J Microbiol Methods* **67**, 310-320 (2006).
11. C. Rodriguez *et al.*, Indirect potable reuse: a sustainable water supply alternative. *International journal of environmental research and public health* **6**, 1174-1209 (2009).
12. J. W. Bisson, V. J. Cabelli, Clostridium perfringens as a water pollution indicator. *J Water Pollut Control Fed* **52**, 241-248 (1980).
13. A. Omarova, K. Tussupova, R. Berndtsson, M. Kalishev, K. Sharapatova, Protozoan Parasites in Drinking Water: A System Approach for Improved Water, Sanitation and Hygiene in Developing Countries. *Int J Environ Res Public Health* **15**, (2018).
14. K. Setty *et al.*, Faster and safer: Research priorities in water and health. *International journal of hygiene and environmental health* **222**, 593-606 (2019).
15. G. N. Stelma, Jr., Use of bacterial spores in monitoring water quality and treatment. *J Water Health* **16**, 491-500 (2018).
16. E. D. Nel, H. Rabie, J. Goodway, M. F. Cotton, A retrospective study of cryptosporidial diarrhea in a region with high HIV prevalence. *J Trop Pediatr* **57**, 289-292 (2011).
17. S. O. Sow *et al.*, The Burden of Cryptosporidium Diarrheal Disease among Children < 24 Months of Age in Moderate/High Mortality Regions of Sub-Saharan Africa and South Asia, Utilizing Data from the Global Enteric Multicenter Study (GEMS). *PLoS Negl Trop Dis* **10**, e0004729 (2016).
18. Ł. Pielok *et al.*, Massive Cryptosporidium infections and chronic diarrhea in HIV-negative patients. *Parasitol Res* **118**, 1937-1942 (2019).
19. F. E. Adeyemo, G. Singh, P. Reddy, F. Bux, T. A. Stenström, Efficiency of chlorine and UV in the inactivation of Cryptosporidium and Giardia in wastewater. *PloS one* **14**, e0216040-e0216040 (2019).
20. V. J. Harwood *et al.*, Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Appl Environ Microbiol* **71**, 3163-3170 (2005).
21. R. M. Chaudhry, K. A. Hamilton, C. N. Haas, K. L. Nelson, Drivers of Microbial Risk for Direct Potable Reuse and de Facto Reuse Treatment Schemes: The Impacts of Source Water Quality and Blending. *International journal of environmental research and public health* **14**, 635 (2017).
22. J. T. Lisle, J. J. Smith, D. D. Edwards, G. A. McFeters, Occurrence of microbial indicators and Clostridium perfringens in wastewater, water column samples, sediments, drinking water, and Weddell seal feces collected at McMurdo Station, Antarctica. *Appl Environ Microbiol* **70**, 7269-7276 (2004).
23. K. Liguori *et al.*, Antimicrobial Resistance Monitoring of Water Environments: A Framework for Standardized Methods and Quality Control. *Environmental Science & Technology* **56**, 9149-9160 (2022).

24. L. Lundy *et al.*, Making Waves: Collaboration in the time of SARS-CoV-2 - rapid development of an international co-operation and wastewater surveillance database to support public health decision-making. *Water Res* **199**, 117167 (2021).
25. Osaigbovo, II, I. O. Igbarumah, E. B. Muoebonam, D. E. Obaseki, Setting up a molecular diagnostic laboratory for SARS-CoV-2 testing: Experience of a single centre in a resource-constrained setting. *Afr J Lab Med* **10**, 1326 (2021).
26. F. Kobia, J. Gitaka, COVID-19: Are Africa's diagnostic challenges blunting response effectiveness? *AAS Open Res* **3**, 4 (2020).
27. S. Watier-Grillot *et al.*, Cryptosporidiosis outbreaks linked to the public water supply in a military camp, France. *PLoS Negl Trop Dis* **16**, e0010776 (2022).
28. E. C. Oriero, J. Jacobs, J.-P. Van Geertruyden, D. Nwakanma, U. D'Alessandro, Molecular-based isothermal tests for field diagnosis of malaria and their potential contribution to malaria elimination. *Journal of Antimicrobial Chemotherapy* **70**, 2-13 (2014).
29. C. Kolm *et al.*, Detection of a microbial source tracking marker by isothermal helicase-dependent amplification and a nucleic acid lateral-flow strip test. *Scientific reports* **9**, 393-393 (2019).
30. I. M. Lobato, C. K. O'Sullivan, Recombinase polymerase amplification: Basics, applications and recent advances. *Trends Analyt Chem* **98**, 19-35 (2018).
31. G. A. Obande, K. K. Banga Singh, Current and Future Perspectives on Isothermal Nucleic Acid Amplification Technologies for Diagnosing Infections. *Infect Drug Resist* **13**, 455-483 (2020).
32. C. Kober, R. Niessner, M. Seidel, Quantification of viable and non-viable Legionella spp. by heterogeneous asymmetric recombinase polymerase amplification (haRPA) on a flow-based chemiluminescence microarray. *Biosens Bioelectron* **100**, 49-55 (2018).
33. Y. Zheng *et al.*, RPA-SYBR Green I based instrument-free visual detection for pathogenic Yersinia enterocolitica in meat. *Anal Biochem* **621**, 114157 (2021).
34. A. Castellanos-Gonzalez, A. C. White, Jr., P. Melby, B. Travi, Molecular diagnosis of protozoan parasites by Recombinase Polymerase Amplification. *Acta tropica* **182**, 4-11 (2018).
35. P. Hou *et al.*, Development of a recombinase polymerase amplification combined with lateral-flow dipstick assay for detection of bovine ephemeral fever virus. *Molecular and cellular probes* **38**, 31-37 (2018).
36. Q. Ma *et al.*, Development of a lateral flow recombinase polymerase amplification assay for rapid and visual detection of Cryptococcus neoformans/C. gattii in cerebral spinal fluid. *BMC infectious diseases* **19**, 108-108 (2019).
37. Y. Peng *et al.*, Rapid detection of Burkholderia pseudomallei with a lateral flow recombinase polymerase amplification assay. *PloS one* **14**, e0213416-e0213416 (2019).
38. A. Saxena, V. Pal, N. K. Tripathi, A. K. Goel, Development of a rapid and sensitive recombinase polymerase amplification-lateral flow assay for detection of Burkholderia mallei. *Transbound Emerg Dis* **66**, 1016-1022 (2019).
39. J. Li, J. Macdonald, F. von Stetten, Review: a comprehensive summary of a decade development of the recombinase polymerase amplification. *Analyst* **144**, 31-67 (2019).
40. S. Kersting, V. Rausch, F. F. Bier, M. von Nickisch-Rosenegk, A recombinase polymerase amplification assay for the diagnosis of atypical pneumonia. *Anal Biochem* **550**, 54-60 (2018).
41. A. Rani *et al.*, Evaluation and comparison of recombinase polymerase amplification coupled with lateral-flow bioassay for Escherichia coli O157:H7 detection using diifeerent genes. *Sci Rep* **11**, 1881 (2021).
42. U. Edwards, T. Rogall, H. Blöcker, M. Emde, E. C. Böttger, Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17**, 7843-7853 (1989).
43. B. Ma *et al.*, A simple and efficient method for potential point-of-care diagnosis of human papillomavirus genotypes: combination of isothermal recombinase polymerase amplification with lateral flow dipstick and reverse dot blot. *Anal Bioanal Chem* **411**, 7451-7460 (2019).
44. K. Lemarchand *et al.*, Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. *Journal of Microbiological Methods* **63**, 115-126 (2005).
45. N. Valeix *et al.*, Multicenter Comparative Study of Six Cryptosporidium parvum DNA Extraction Protocols Including Mechanical Pretreatment from Stool Samples. *Microorganisms* **8**, (2020).
46. V. P. Edgcomb, J. H. McDonald, R. Devereux, D. W. Smith, Estimation of bacterial cell numbers in humic acid-rich salt marsh sediments with probes directed to 16S ribosomal DNA. *Appl Environ Microbiol* **65**, 1516-1523 (1999).
47. O. Piepenburg, C. H. Williams, D. L. Stemple, N. A. Armes, DNA detection using recombination proteins. *PLoS Biol* **4**, e204 (2006).
48. L. Lillis *et al.*, Factors influencing Recombinase polymerase amplification (RPA) assay outcomes at point of care. *Mol Cell Probes* **30**, 74-78 (2016).

APPENDIX A: MATERIALS USED IN STUDY

1. Water samples
2. Filters (0.22µ filters) and syringes (cheaper option); will also investigate ready-made beakers with filters (these required a vacuum pump, thus ideal for a laboratory, but not for fieldwork where electricity supply might be problematic).
3. TwistDX kits
4. Primers (use primers from Inqaba Biotec – supplied within one week, primers from IDT usually supplied within one month but reportedly better quality).
5. Culture media
6. Blood agars and McConkey from Greenpoint Media (NHLS)
7. Buffers Charcoal Yeast Extract Agar (for *Legionella*)
8. Anaerobe culture supplies
    a. Anaerogen sachets – used to generate anaerobic conditions inside boxes for organisms that need the absence of oxygen for growth (such as *C. perfringens*)
    b. Culturing for *C. perfringens* requires anaerobic conditions – we will evaluate the use of anaerobic sachets and anaerobe culture boxes; can also do in an anaerobe workstation functioning with gas – the latter is expensive option and not available in most laboratory in South Africa. Such a workstation is available in the host department of UCT where study is done and will be used in the study.
9. Phosphate buffered saline.
10. Swabs

APPENDIX B: BRIEF DESCRIPTION OF KITS USED

B1: DETECTION OF PATHOGEN DNA USING LABELLED AMPLICONS AND A LATERAL FLOW DEVICE.

Workflow for proof of principle

Tenfold serial dilutions of extracted genomic DNA were made for the 3 pathogens *E. coli* 0157:H7, *C. perfringens* and *Legionella pneumophila* (serogroup1).

The range was 10 ng to 10x 10^{-6} ng/µl of DNA. These were used for amplification of relevant targets, i.e. the *rfbE*, *mip* and *plc* genes of the respective pathogens mentioned above. These genes are unique to the relevant pathogens and commonly used in the literature to detect these specific pathogens amongst closely related strains or species.

To enable detection using a lateral flow device with binding capabilities of amplicons labelled with either DIG or 5-FAM, and streptavidin- based detection (by interaction of streptavidin and biotin), all reverse primers for the targets were labelled with biotin, while the forward primers were labelled with 5-FAM in the case of *E. coli*, and biotin for *L. pneumophila* and *C. perfringens* respectively. *E. coli* DNA should be detected on test line2 of the strip, while the other 2 bacteria should be detected on test line 1.

Both conventional PCR (using heat to separate double stranded DNA and various temperatures to do amplification) and recombinase polymerase amplification (using a proprietary recombinase to separate DNA strand and DNA-binding proteins to keep DNA single stranded, plus amplification at one temperature for entire process) was done. Amplicons were detected using both agarose gel electrophoreses as well as lateral flow strips (the PCRD device, Abingdon Health UK).

Materials

Relevant bacterial cultures (used pure ATCC reference strains).

DNA extraction kit

OneTaq PCR Readymix for conventional PCR, TwistAmp Basic kit for RPA PCR, labelled primers to generate double labelled amplicons.

2% agarose gels prepared in 1x TAE buffer and UV detection of fluorescent DNA on gel in the presence of ethidium bromide.

Various DNA molecular weight markers, e.g. 100bp Quickload DNA ladder (NEB)

For PCRD lateral flow device, line 1 will detect amplicons labelled with DIG (*C. perfringens* and *L. pneumophila*).

Line2 will detect amplicons labelled with 5-FAM or FITC (*E. coli* 0157:H7).

See online PCRD resources to see how device works: https://www.abingdonhealth.com/products/pcrd/

For LFD detection: As per manufacturer instruction,84ul of diluent from kit was mixed with 6ul of PCR amplicons. This was mixed and loaded onto the strip. Results was read within 10minutes and not thereafter, as instructed.

RPA PCR was done at 37^{o}C, for 30minutes, in a PCR machine.

The principle of the lateral flow detection is described on the manufacturer's website, with a video to demonstrate: https://www.abingdonhealth.com/products/pcrd/.

B2: QUALITY CONTROL: ALSO TESTING IF THE NEGATIVE CONTROLS FROM CONVENTIONAL PCR WOULD SHOW AN LFD SIGNAL

Tested if the no template control PCR (NTC) will generate background on the strips (in other words if unincorporated or primers or dimers detected).

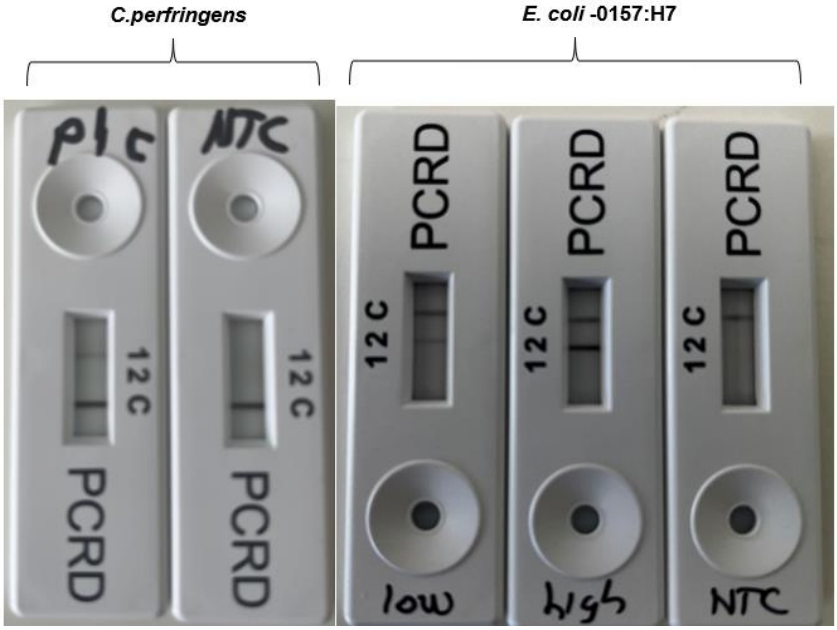

Figure B1. Detection of *C. perfringens and E. coli* 0157:H7 DNA using a lateral flow device.
The NTC = negative controls for the PCR. Thus, it does not detect unincorporated primers left over from conventional PCR. Low = about 5 ng input DNA, high= about 30 ng input DNA. Test line 2 detect *E. coli*, test, with 5-FAM labelled amplicons, line 1 detects *L. pneumophila or C. perfringens,* both with DIG labelled amplicons. C=control line and is quality control that device is working.

B3: COMPARISON OF DNA EXTRACTION KITS

Peer reviewed published literature was searched for kits used for DNA extractions for environmental samples. 3 kits were selected for testing in this project, with a 4th kit under investigation, but not tested yet with RPA PCR. The kits used are compared in Supplementary Table 1 below; we opted for kits using different clean-up methods and not one specific DNA clean-up method.

SUPPLEMENTARY TABLE 1. COMPARISON OF DNA EXTRACTON KITS USED IN STUDY.

KIT NAME	**Mag-Bind® Environmental DNA 96 Kit (Omega Biotek)**	QuickDNA Fecal/Soil Microbe Miniprep Kit (Zymo Research)	Wizard Genomic DNA Kit (Promega)	PrepGem Bacteria (Microgen)
Cold storage required?	For lysozyme, not kit reagents	For lysozyme (or Proteinase if step needed), not kit reagents	For lysozyme, not kit reagents	Yes
CLEANUP METHOD	Magnetic beads	Spin column	Chemical, "protein precipitation"	No purification
Cell lysis and homogenization	Ceramic Beads Vortex	Ceramic Beads Vortex	No beads Vortex	No beads Vortex
Adsorption of inhibitors	Proprietry cHTR reagent -remove humic acid and unspecified other PCR inhibitors.	Not listed in reagents	Not listed in reagents	Not listed in reagents
Approximate time to completion	90 to 120 min	Max 80 min	45 to 60 min	30 to 40 min
Average cost of kit[4]	Above 10K ZAR	Below 10K ZAR	Below 10K ZAR	Below 10K ZAR
Additional reagents not supplied by kit	Ethanol	Ethanol	Ethanol	
Additional equipment	Heated device capable of 70ºC and below Microcentrifuge Vortex Heated device capable of 70ºC Microcentrifuge Cell disruptor device ("bead beater") Magnetised device to facilitate the extraction via magnetic beads. Sterile Plastic consumables	Heated device capable of 70ºC and below Microcentrifuge Cell disruptor device ("bead beater") Sterile Plastic consumables	Heated device capable of 70ºC and below Microcentrifuge Cell disruptor device ("bead beater") Sterile Plastic consumables	Heated device capable of 70ºC and below
COMMENT	Long protocol, multiple vertexing steps a concern for DNA integrity (could verify if less steps efficient). Ideal for water studies, but the extensive nr steps will increase chances of technical mistakes. Automation better option, to avoid human error.	Efficient and fit for purpose, but high bioburden releasing large amount of DNA will clog the columns. Will need to optimise how much elution buffer to use for samples with different bioburden	Efficient, but DNA precipitation and waiting period for DNA dissolving will extent TAT. Very easy to use, if large amount of DNA, one can see it spooling out during precipitation step.	Not explored in this project, just preliminary testing to see if it effectively lyses *Clostridium perfringens*