Development of a Rapid Molecular Method for detection of *Clostridium perfringens* and *Legionella pneumophila* in water sources.

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MSc Candidate

Naqsha Savahl

SVHNAQ001

Under the supervision of

Dr. Lynthia Paul and Dr. Nisreen Hoosain

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ABBREVIATIONS

BCYE	Buffered Charcoal Yeast Extract Agar
BHI	Brain-heart infusion
BLAST	Basic Local Alignment Search Tool
CCLs	Contaminant Candidate Lists
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DIG	Digoxigenin
dsDNA	Double stranded DNA
EMA	Ethidium monoazide
GVPC	Glycine, Vancomycin hydrochloride, Polymyxin B sulphate, Cycloheximide
gDNA	Genomic DNA
ID	Identification number
LFA	Lateral Flow Assay
LFD	Lateral flow detection
LFICA	Lateral Flow Immunochromatographic Assay
LMICs	Low and middle income countries
IFT	Immuno Fluorescence Test
MIP	Macrophage infectivity potentiator
NICD	National Institute for Communicable Diseases
RPA	Recombinase Polymerase Amplification
RPA-LFA	Combination of Recombinase Polymerase Amplification and Lateral Flow Assay
PLC	Phospholipase gene of *Clostridium perfringens*
POC	Point of care
PCR	Polymerase Chain Reaction

PMA	Propidium monoazide
qPCR	Quantitative PCR
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
SAMRS	Self-avoiding molecular recognition system
SDWA	Safe Drinking Water Act
US EPA	United States Environmental Protection Agency
VBNC	Viable but not culturable
WBD	Waterborne diarrhoea
WASH	Water, sanitation and hygiene
WWTPs	Wastewater treatment plants

ABSTRACT

Access to clean water is a basic requirement for all. Global water scarcity necessitates the reclamation and reuse of water. Effective, rapid surveillance for pathogens in recycled water is essential, assisting pathogen detection in water intended for reuse by humans, animals, and industry. Monitoring of repurposed water is essential as it might contain residual microorganisms that are less sensitive to disinfection. Examples are *Clostridium perfringens* (a spore forming microorganism with a cell envelope that is less sensitive to chemical disinfection and the cause of diarrhoea via contaminated water and food) and *Legionella pneumophila* (a waterborne, pulmonary pathogen protected from disinfection due to its intracellular lifestyle inside Protists such as *Entamoeba*). Molecular detection of nucleic acids is a tool enabling more rapid detection of pathogens in water compared to culture-based methods. Molecular tests using nucleic amplification and detection via the polymerase chain reaction (PCR) method needs thermal cycling instruments and thus is limited in resource-poor environments and spaces lacking infrastructure such as electricity. A solution is to use isothermal amplification methods, requiring heated devices at a single temperature and excluding the need for thermal cycling devices. A battery-operated heated device suffices for isothermal methods such as Recombinase Polymerase Amplification (RPA) and requires less time compared to PCR. Furthermore, isothermal amplification, when combined with the detection of generated amplicons on lateral flow devices, excludes the need for detection methods requiring electrified equipment. The study described here aimed to evaluate RPA, combined with lateral flow detection of labelled amplicons, as a tool to detect deoxyribonucleic acid (DNA) from two waterborne pathogens, i.e., *Clostridium perfringens* (*C. perfringens*) and *Legionella pneumophila* (*L. pneumophila*).

In-house designed primers targeting the *C. perfringens* phospholipase C gene (*plc*) and previously published primers to detect *L. pneumophila mipA* gene were evaluated using conventional PCR and RPA. The lowest amount of DNA needed for amplification and detection was determined using PCR and both agarose gel electrophoreses and lateral flow detection (LFD). For LFD, labelled primers were used to generate dual-labelled amplicons that were captured and detected on a commercial lateral flow device. The RPA-LFD method was then tested with DNA extracted from a limited number of water samples provided by the local municipality. Lastly, as efficient DNA extraction is a limiting step in molecular detection. Consequently three commercial, manual DNA purification methods were evaluated for their ability to extract DNA from *C. perfringens.*

The generated data showed successful detection of DNA from both pathogens via RPA-

LFD). A minimum of at least $1x10^{-3}$ ng of pure DNA, extracted from the cultured pathogens, were efficiently amplified, and detected for both microorganisms. Future work should evaluate the method more extensively with DNA extracted from variety of real-world water samples as PCR inhibitors in water from different resources might influence the sensitivity of the method. Regarding DNA extractions, we compared three different DNA extraction and purification methods (solid phase, desalting and magnetic bead purification) to obtain fit for purpose DNA from cultured *C. perfringens*. Extraction kits were also compared for equipment and infrastructure needed, efficiency of extraction, cost, and ease of use. All kits generated DNA fit for downstream amplification, but consideration should be given to kits that require fewer steps to obtain pure DNA and use minimal equipment. Kits with a removal step for PCR inhibitors in water should also preferentially be used.

In conclusion, the data shows that RPA-LFD could be a feasible method to monitor pathogens in water. Further development of the RPA-LFD method using dual labelled amplicons are necessary, including validation of the method with an expanded set of real-world water samples. The method should further be combined with viability testing, to confirm that DNA detected from samples originates from viable microorganisms and not cell free DNA present in water.

INTRODUCTION & PROBLEM STATEMENT

At least two billion people are impacted by the global water crisis, drinking from water sources with faecal contamination and living in water stressed areas (1). Contaminated water is associated with an estimated 505 000 annual diarrhoea-related deaths (2). Increases in population growth, urbanization, ongoing climate changes and increases in water scarcity impose a greater strain on water systems (2–4). Other sources for drinking and irrigation water such as groundwater, and wastewater are therefore increasingly being used or considered as alternative sources (2).

Waterborne pathogens include bacterial, viral, fungal, and protozoan microorganisms responsible for a variety of infectious diseases, including diarrhoea, respiratory disease and septicaemia. Well known examples include bacterial species *Escherichia coli* O157:H7, *Clostridioides difficile* (5,6), *Campylobacter* spp. (7) and *Clostridium perfringens* (8,9), known diarrhoeal pathogens that could originate from contaminated water sources. In the United States of America, a country with good surveillance systems, 207 waterborne disease outbreaks were detected over a 9-year period prior to and including the year 2002 (10). Importantly, this type of surveillance is mostly done in first world nations with adequate resources and not done to the same extent globally, lacking many countries, notably resource poor countries.

Due to is the scarcity of water, alternative sources such as reclaimed water are increasingly utilised for economic, animal, and human recreational purposes. However, the use of reclaimed water sources is a potential health risk, notably the transmission of residual waterborne pathogens if the water quality of these resources is not monitored and managed appropriately (11). No local guidelines currently exist to govern the safe use of reclaimed water. Currently, global microbial water quality assessments use *Escherichia coli* (*E. coli*) as the benchmark to determine the suitability of water for human use. However, *E. coli* is not an ideal indicator for microorganisms that are less sensitive to inactivation. These include thermotolerant, intracellular living *Legionella* or spore-formers such as *C. perfringens* and protozoans such as *Cryptosporidium*. These resilient pathogens could cause infections, if viable, residual spores or vegetative cells remain in treated water destined for reuse. For example, *C. perfringens,* is associated with self-limiting, foodborne and waterborne gastroenteritis (12,13), as well as tissue-destructive, lethal soft tissue infections (14). This pathogen is widely distributed in the environment and can be transmitted by humans as well as animals (15–17). Catchment soils are another reservoir that seeds downstream water (18). Thus, contamination of open wounds and food might

occur from the environment, including contaminated water used for cooking.

Waterborne pathogens responsible for respiratory disease include several species of *Legionella*. Notably, the presence of *L. pneumophila* in repurposed water and water from ageing infrastructure (19) is an increasing global concern (20,21). Members of this genus are present in water distribution systems, air-conditioning devices, spa baths and cooling towers (22). In countries with good surveillance data, such as the USA, there is evidence of waterborne outbreaks associated with *L. pneumophila*, while its presence in wastewater is understudied and most likely underestimated (23). Wastewater treatment facilities may also be the cause of local or community transmission of *Legionella* (23). As treated wastewater is discharged into natural water systems or reused after treatment, it could thus pose a risk for disease. The aerobic treatment of wastewater provides the ideal temperature, oxygen availability and organic nitrogen while the presence of protozoa provides ideal conditions for the growth of *Legionella* (23). Furthermore, the presence of *Legionella* in ageing buildings with old water infrastructure is problematic. In South Africa specifically, *Legionella* has also been detected in potable water sources within a public healthcare facility. In the reporting study, a mixture of culture and molecular detection of nucleic acid DNA was used and showed the disparity between culture-based and molecular methods to reliably detect the presence of this microorganism (18). This has a bearing on the public health care risk posed by microorganisms in ageing water systems. However, despite Legionellosis being a notifiable disease in South Africa, is still rarely reported (24).

Due to an increased demand in for water and sanitation services, testing technologies such as the detection of microbial nucleic acids are necessary to ensure effective, rapid water quality monitoring, ensuring that water is fit for human consumption, recreation, irrigation, and industrial use. Molecular methods not only simplify the monitoring of microbiological water quality determinants, but also provides faster turnaround times for test results (8,25). For instance, molecular surveillance of the severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) in sewage, wastewater and other systems was highly effective during the height of the COVID pandemic (26,27). Molecular technologies such as PCR, real-time PCR, isothermal amplification methods (such as loop-mediated isothermal amplification) and enzyme-linked immunosorbent assays have successfully been used to predict outbreaks of Covid-19 by analysing wastewater (28,29).

Molecular based detection is more feasible for a variety of reasons. For example, not all viable bacteria are culturable (thus will generate false negative diagnostic results), while the disparity between growth rates of different microbes could result in fast growers

outcompeting and outgrowing the slower growing pathogens (30). Furthermore, some bacteria require specialised media and chemicals for growth, which is not cost effective in resource limited settings. All these factors influence diagnostic turnover time, cost, and outcomes. On the other hand, molecular detection is rapid, detects nucleic acid signatures and can be combined with assays to prove the viability of target microorganisms. The latter, so-called “viability PCR” (using dyes to distinguish between DNA from live and dead bacteria, ensuring that only live microbes are detected), is an area requiring further development. Methods to detect generated amplicons of targeted microorganisms include real-time PCR to detect fluorescence in real-time, traditional gel electrophoreses combined with ultraviolet fluorescence detection, as well as lateral low detection of labelled amplicons. The latter is rapid and more feasible in situations where the use of electrical equipment is problematic. This is relevant for resource poor countries and situations where tests need to be done in remote settings far from laboratories. In these situations, a field laboratory, using mobile equipment powered by batteries and molecular workflows requiring minimal specialised equipment is ideal. In this regard, a workflow using DNA amplification and detection of amplicons via handheld lateral flow devices is more feasible for the remote detection of pathogens and requires less technical expertise. Notably, the availability of lyophilized, ready-made amplification mixes excludes the need for cold chain management; to date no products on the market enable this. Yet to date, limited studies have been conducted on the detection of waterborne pathogens using POC (point of care) or near POC workflows that use minimal, specialised equipment to detect waterborne pathogens (31).

STUDY RATIONALE

As the increasing demand for safe water sources necessitates rapid, reliable, and effective water quality monitoring, molecular methods requiring minimal infrastructure, instrumentation and technology would improve effective water quality monitoring. Isothermal amplification methods and detection of DNA amplicons using paper based lateral flow detection are ideal/cost effective for resource limited and remote settings.

This study aimed to evaluate an isothermal nucleic acid amplification method that enables the generation of DNA amplicons at a single temperature (below 45 ˚C). We also aimed to combine the isothermal method with LFA detection of the generated amplicons. LFA methodology will enable molecular detection of pathogens in water sources without the use of real-time PCR equipment. Although not in the scope of this project, the method tested in this study ideally should be combined with confirmation of bacterial viability.

CHAPTER 1 LITERATURE REVIEW

The 2030 Agenda for Sustainable Development was adopted by all United Nations Member States in 2015. It presents a shared blueprint for global prosperity and peace that considers both the present and the future needs of people and the environment. There are 17 Sustainable Development Goals (SDGs) which are an urgent call for global partnership between all nations. SDG 6 focuses on ensuring the availability and sustainable management of water and sanitation for all. This literature review will first discuss the global impact of access to clean water, then provide insights on current gold standards for testing and limitations thereof, followed lastly by an extensive review of rapid testing methodologies that could contribute to improved surveillance and assurance of water quality.

Access to safe clean water is essential for public health and sustainability.

Diarrhoea remains a significant global burden of disease and mortality, being the 8th leading cause of global mortality, with an estimated 1.5 million deaths, reported in 2019. This reflects a decrease from 2.6 million deaths reported in 2000 (32). Approximately 15% of all under-five fatalities worldwide were caused by diarrhoea. Seventy-eight percent of reported paediatric diarrhoea deaths occurred in Southeast Asia and Africa, specifically in low and middle income countries (LMICs). Without the necessary action, it is predicted that infectious diseases will claim the lives of 4.4 million children under the age of five each year by 2030, with 60% of those fatalities happening in sub-Saharan Africa (33). In Africa, informal settlements are often associated with public health issues due to their dense population and inadequate sanitation (34).

In South Africa waterborne diarrhoea (WBD) statistics are released by the National Institute of Communicable Diseases (NICD), and data from waterborne outbreaks reported in regular bulletins illustrate that WBD is an ongoing issue in the country (35). For example, one 2019 issue of the relevant communique reported a spike in diarrhoeal cases in one town, with norovirus and *Giardia* observed in patient faecal samples. Concurrently, an investigation of the water source showed that water provided to the relevant town was not compliant with SANS 241:2015 standards for drinking water (as evidenced by levels of *E. coli* above accepted standards) (36). However, a retrospective study done by Malebatja and Mokgatle (2022), using municipal data, concluded that drinking water quality was not correlated to morbidity and mortality rates amongst South African children under the age of five in the period 2010-2018. These authors used plant data from municipalities across South Africa, collected earlier than the 2019 Giardia outbreak (37). The authors concluded that local

drinking water complied with the standards for drinking water as guided by the SANS 241:2015 (37).

Urbanisation, plus new and expansion of existing informal settlements is a risk factor contributing to infectious disease. With a projected 2.40% annual growth rate, Cape Town's population was estimated to be 4,617,560 in 2016, with 17% of those people living in unofficial housing. Numerous of these informal settlements are situated on steep hillsides or low-lying, flood-prone areas with inadequate water and sanitary facilities. These informal settlements are typically characterised by crowded living quarters, unhygienic surroundings, inappropriate waste disposal, and inadequate sanitation. Due to these living circumstances, residents in those places are especially susceptible to diarrhoea (38). This is of particular concern since diarrhoea is noted to be the eighth leading cause of mortality in South Africa (both sexes and not aged restricted) (Fig. 1.1) and the fifth leading cause of mortality in the under aged five group (both sexes) (Fig. 1.2) (39). In Cape Town (situated in the Western Cape Province of South Africa) data from the Department of Health indicates that diarrhoea varies seasonally, peaking each year between March and June (Fig. 1.3) (40). The number of cases appears to increase: in November 2022, 4133 cases of diarrhoea were reported in children under age five, which increased to 44412 cases in 2023 (41). However, Global Burden of Disease (GBD) data suggests a decline in mortality due to diarrhoea in South Africa (Fig. 1.4).

The abovementioned cases are in line with global observations; according to the World Health Organization, exposure to contaminated water remains one of the main drivers of diarrheal outbreaks.

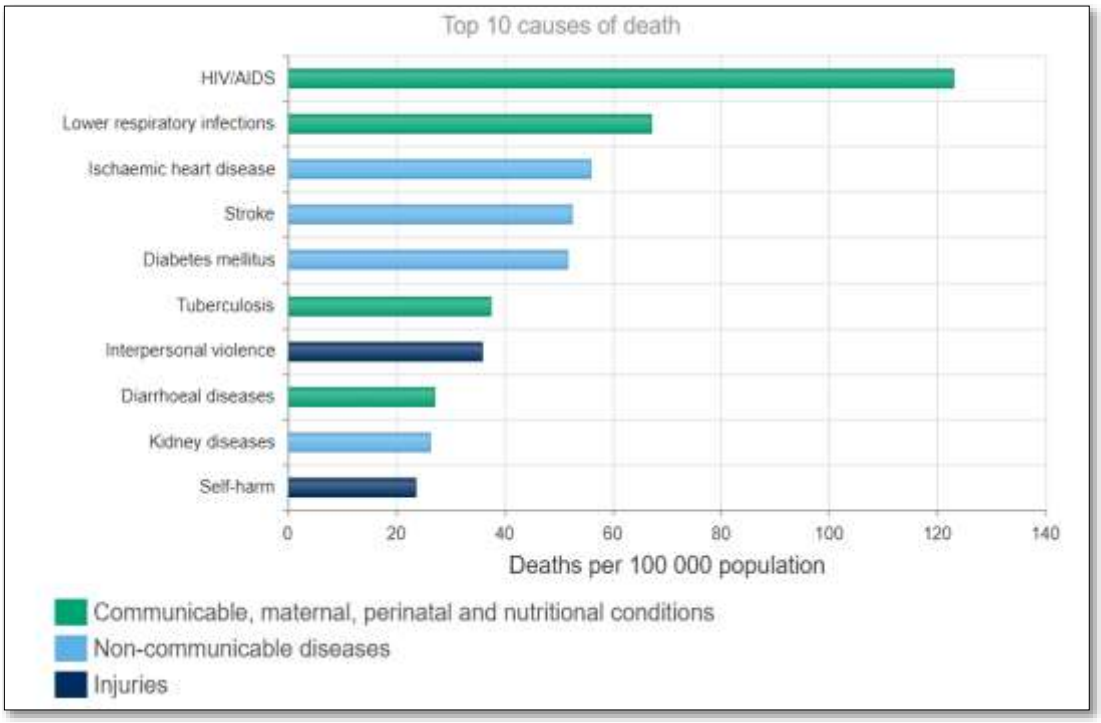

Figure 1.1. Top 10 causes of death in South Africa for both sexes and all ages (2019).

Data depicting top 10 causes of death where diarrhoeal diseases account for 20 per 100 000 of the population. Figure and data obtained from: [Global health estimates: Leading causes of death (who.int)](#) (42).

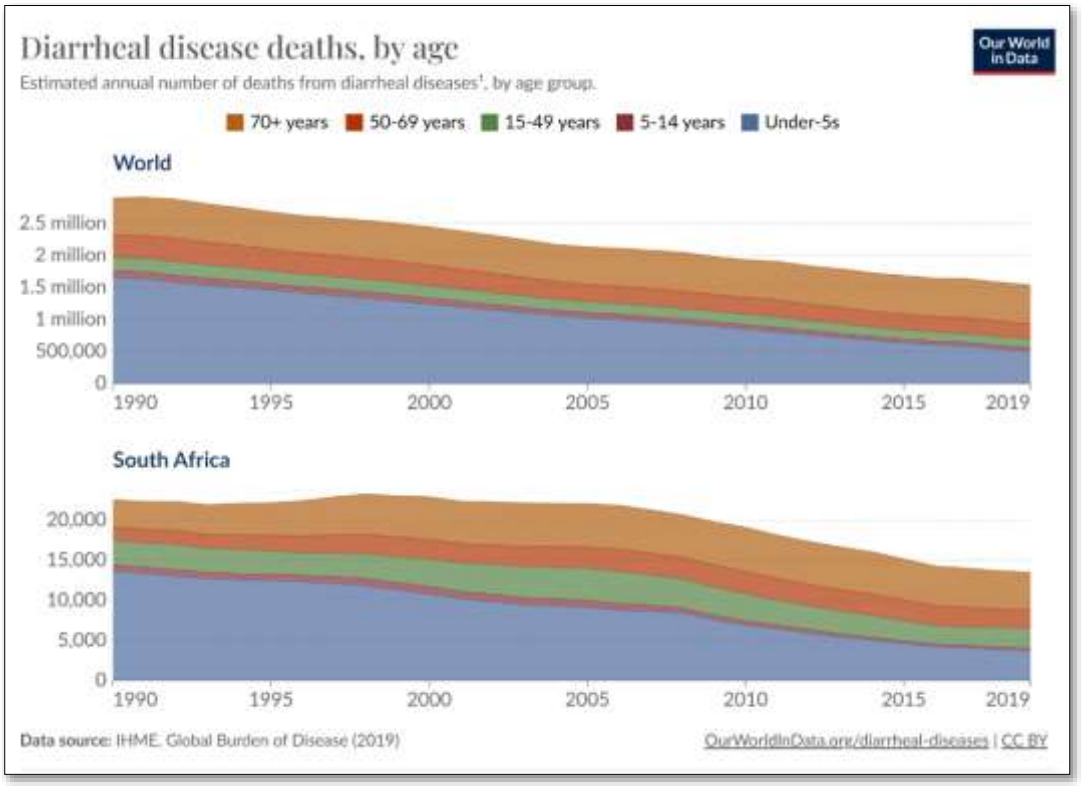

Fig. 1.2. Global and South African mortality depicting by age from infectious diarrhoeal disease (1990-2019).

As indicated in the graph the death decreased from 1990-2019 both globally and in South Africa. Figure from Our World in data (39)

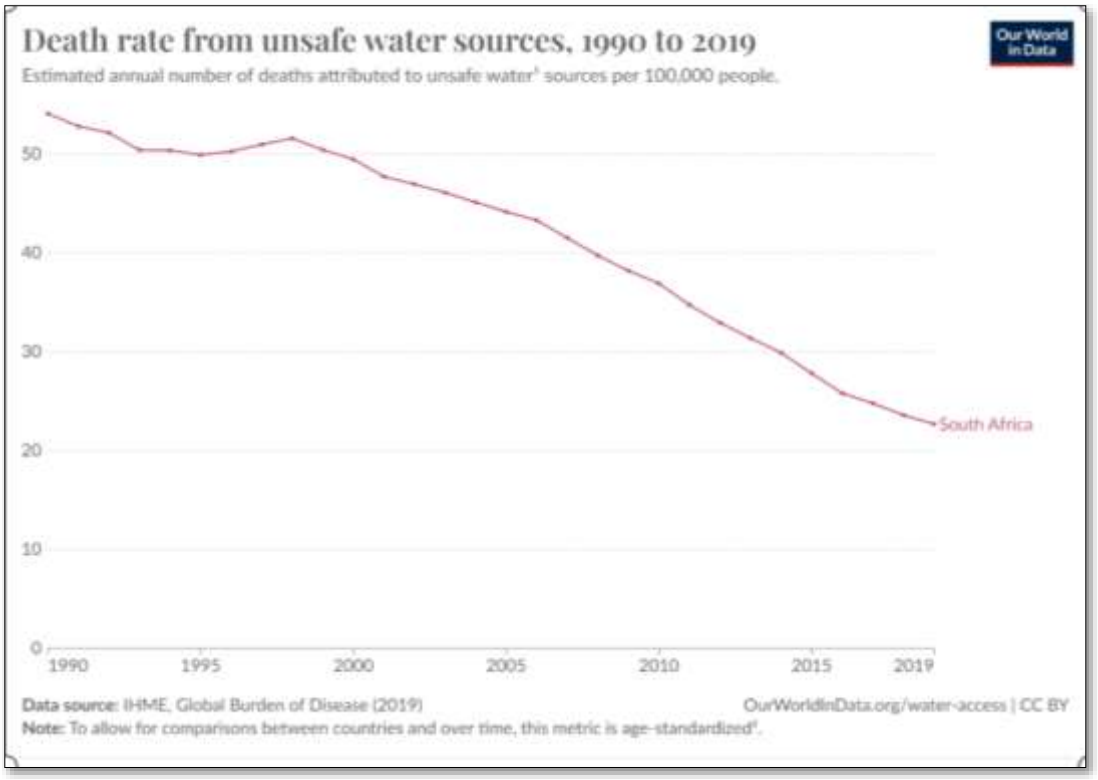

Fig. 1.3. Mortality in South Africa attributed to unsafe water sources during 1990-2019. As indicated the death rate from unsafe water sources declined from 1990-2019 (40)**.**

The abovementioned data (Fig. 1.1-1.3) underscores that safe water needs to be a global priority. As mentioned before, global water systems are under severe strain and are negatively impacted by population growth, rapid urbanization, climate change, drought, natural disasters (earthquakes, tropical storms/cyclones, tsunamis etc.) and political instability (such as wars). Other factors such as ageing sewage infrastructure, sewer overflows, non-compliant effluents due to electricity load shedding and blackouts and the proliferation of informal settlements around water sources such as rivers and dams are also major contributors to faecal pollution of water (42–46).

Prospects are considerably worse on a global scale, since it is predicted that half of humanity will live in water-stressed areas by the year 2025, stressing that urgent measures are essential to counter the potential impact this will have on health and sustainability. These measures should include prevention, monitoring and effective responses to potential health threats posed by contaminated water. Education and political will, t to drive optimal surveillance and prevention measures would further ensure the provision of safe water to everyone. For example, the positive impact of initiatives such as WASH (water, sanitation and hygiene) interventions are well studied and reported, as shown in a recent systematic

review (47). WASH data generated from 124 studies showed that WASH interventions significantly reduced the risk of diarrhoea in places where WASH was implemented (47).

Water quality monitoring

Solutions to improve the global situation are a priority; therefore, resources are channelled towards finding alternate sources for drinking and irrigation as well as improved detection of pathogens in water. Improved detection and surveillance should use methodology fit for purpose. The specific environment determines the type of instrument detection methodology and negatively impacts surveillance and diagnostics in resource constrained environments. Notably, turnover time is significantly impacted by available infrastructure, technology, and expertise.

Furthermore, water quality must be monitored and managed appropriately, with compliance with national and international quality standards such as the South African National Standards 241:2015 (SANS, 2015), WHO drinking water guidelines (2022) and EU Council Directive 98/83/EC (1998) (2,48). Other guidelines, such as the Centre for Disease Control (CDC) and the latest US EPA Contaminant Candidate List, t should also be consulted to be informed on the microbes that have been identified as health risks or with the potential to cause harm. The CDC website contains a list of top microbial contaminants associated with disease upon consumption of inadequately treated tap water (49). On the other hand, the US EPA publishes updated Contaminant Candidate Lists (CCLs) where the organization lists contaminants (microbial and chemical) that are currently not subjected to any regulatory drinking water guidelines but are known or thought to occur in public water systems. It should be noted that contaminants that are listed on the updated CCL may require future regulation under the Safe Drinking Water Act (SDWA) if extensive research data warrants the decision (50).

Using standardised guidelines, combined with political will and incentives to ensure the safe delivery of water to populations, s will contribute to a decrease in diarrheal disease, as was shown in the previously mentioned WASH report (47).

If alternate sources are the solution in a specific setting, these alternate sources of water should be surveyed effectively to prevent disease outbreaks. Alternate resources targeted for use include groundwater, and reclaimed water (2). Unfortunately, these alternate sources are a public health risk as they could contain bacterial, viral, and protozoan pathogens of faecal origin (51,52). Bacterial pathogens of relevance include members of the genera *Escherichia*, *Burkholderia*, *Campylobacter*, *Legionella*, *Francisella*, non-tuberculous

mycobacteria, *Salmonella*, *Shigella*, *Clostridium* and *Vibrio* as well as *Arcobacter* (53,54).

Current gold standard for pathogen surveillance and detection

Monitoring laboratories employ both culture-based and molecular-based methods to detect the presence of pathogens. In general, *E. coli* counts are employed to detect the presence of faecal contamination of drinking water, while also used to do surveillance of water systems (54). Total coliform counts are a useful predictor of the cleanliness and integrity of the water distribution network (55).

Other proposed indicators including C. perfringens, have been suggested to monitor the efficacy of disinfection and removal of more hardy microorganisms such as *Cryptosporidium*, *Giardia*, helminth eggs and protozoans (56). Also, knowledge of ecology and physiology of waterborne pathogens is are essential. Notably, intracellular pathogens and those with different life stages (which include a sporulation phase) or intracellular lifestyles are more resilient. For example, *Legionella* species can multiply quickly and intracellularly within protozoan hosts for extended periods of time, generating amoebic vesicles that can hold hundreds of *Legionella* cells (18). The intracellular lifestyle of *Legionella* is of particular concern, as its hosts are also known to survive in water infrastructures. Aerosols carrying the bacteria are breathed or aspirated by a vulnerable host, resulting in infection (57). Water from ageing systems poses a particular risk. For example, a research group reported on DNA detection of *L. pneumophila* in water pipes in a specific South African clinical setting. Interestingly, cultures were negative, but the positive PCR tests were correlated with the presence of certain amoeba; both of which present a public healthcare risk (18). This data suggests that culture-based detection for this microorganism is not optimal and yields false negative results, an observation also confirmed by other researchers (58–61). Studies conducted by Nisar et al., (2023) further indicated that the pre-treatment procedure required by culture-based detection method ISO11731:2017 causes underestimation of *Legionella* (61). Thus, molecular detection of nucleic acid or detection of microbial antigens would be a solution. However, antigens from intracellular pathogens will not be detected, leaving the detection of nucleic acids as the most plausible solution.

The Directive (EU) 2020/2184 indicates that the level of *C. perfringens* in water should be 0 cfu/100ml and should only be assessed if the risk assessments indicate that it is appropriate to do so (62). In healthy individuals, the infective dose for most enteric bacteria are reported to be greater than 10^4 viable cells (63) while for *C. perfringens* the infective dose is $>10^6$ vegetative cells or spores/g of food (64). However, according to the abovementioned guideline, zero residual spore-forming micro-organisms is acceptable and any present in

repurposed water poses a threat to public health as some of these micro-organisms are generally more resistant to chemical treatment (65). This strict guideline is likely related to the fact that the presence of clostridial spores is indicative that other resilient organisms are also potentially then present. It is also important to note that inadequate treatment and residual chlorine levels in water can lead to outbreaks of diarrhoea and respiratory diseases, even for less hardy organisms and not only spore-formers such as *C. perfringens*(66)(67) are examples of outbreaks occurred due to low residual chlorine levels in the water. In the Delmas outbreak, a waterborne outbreak of typhoid fever was due to potential poor water quality. In the case of the city of Flint (USA), a water supply change was considered the reason for an outbreak of Legionnaires' disease that killed 12 people and affected 87 more cases. Thus, using hardy microorganisms such as *C. perfringens* as additional indicators of potential contamination of treated water with other microbes is useful, although this microorganism currently is not universally used as an indicator.

The spore-former *C. perfringens* is responsible for toxin-induced non-lethal gastroenteritis (64), potentially lethal soft tissue infections, gas gangrene and neonatal necrotizing enterocolitis (67,14). Known to persist in environments such as soil, environmental waters and catchment soils, the microbe is also harboured by mammals and birds (15,68,69). Once spores are ingested or contaminate open wounds, the actively growing vegetative cells multiply and again sporulate in the small intestine or infected tissues of humans and animals (70). The conditions inside the intestinal tract induce the sporulation of vegetative cells; these spores reach the environment via excrement and subsequently reach water bodies.

Community acquired infections might result in higher pathogen loads being released into the sewage system (68,71) indicating that *C. perfringens s*pores may be a more suitable indicator for faecal contamination due to its wide distribution in sewerage. Although spores are unable to multiply in water, their residual presence may indicate prior faecal contamination of the water supply (14,72). Due to the resistance of spores to chemical disinfectants and ultraviolet light (which are common water treatments), some spores may still be present after treatment (73).

Several research groups have suggested the use of *C. perfringens* spores as a possible surrogate for environmentally stable pathogens such as bacteria, protozoan cysts and specific life-stages of some pathogens e.g. oocysts (14,71,74,75). Inadequate filtration is a known cause of contaminated water supplies, resulting in outbreaks of *Cryptosporidium* (76–78). *Cryptosporidium* oocytes are smaller in size compared to *C perfringens* spore., Thus presence of *C. perfringens* spores after filtration, may indicate that treatment was

inadequate and suggestive that the water may also contain protozoon parasites such as *Cryptosporidium*, enteric viruses and/or protozoa (71,79,80). However, conclusive evidence that *C. perfringens* spores can be used as a reliable indicator for enteric viruses is lacking and data to date is inconsistent. Thus, the WHO has recommended a cautionary approach for the association of *C. perfringens* spores and enteric viruses (2).

Despite the abovementioned limitations and concerns, the increased need for water (for human, recreational, farming, and industrial activities) and sanitation services demands the use of non-traditional water sources (as mentioned earlier in this review), and thus efficient, rapid monitoring of such sources. To counter the time constraint of traditional culturing and phenotyping, rapid testing technologies are necessary to validate good water quality intended for human consumption, recreation, and irrigation. Rapid testing had been demonstrated to improve surveillance compared to current culture-based methods to detect pathogens in water (25). Rapid methods include detection of microbial proteins, metabolic signatures or nucleic acids (81). Molecular-based methods detecting nucleic acids include conventional Polymerase Chain Reaction (PCR) and real-time/quantitative PCR (qPCR). More recently, isothermal amplification methods also are increasingly used.

Amplicons generated by conventional PCR and isothermal amplification methods are generally detected using agarose gel electrophoresis, while amplicons from real-time PCR are detected via fluorescence. For the latter, either the entire amplicon is labelled (during PCR) with a dye such as SYBR-I, or a fluorescently labelled probe is detected once bound to generated amplicons. Both methods are valuable tools for rapid pathogen detection in various sources, including water, through real-time PCR. The advantages and disadvantages of conventional and real-time PCR are shown in Table 1.1. The next section of this review will focus on molecular detection of microbial signatures in water.

Molecular detection of microbial nucleic acids in water

In general, surveillance is done using polymerase chain reaction (PCR), either conventional or real-time amplification of nucleic acid, which is used by the majority of laboratories. Recently developed isothermal amplification methods are also increasingly used (82). These amplification methods typically involve the amplification of DNA or RNA targets in a sample, followed by the detection of generated amplicons. Detection of generated amplicons is done by UV fluorescence of amplicons on a gel or fluorescently labelled amplicons using computerised detection methods and software.

Typical PCR requires heat above 94 °C to denature the double stranded target DNA into

two single strands, giving access to primers to bind to specific sequences of the single stranded DNA. This initiates DNA synthesis via thermostable DNA polymerase at a lower temperature (72 °C), and the four nucleotides as well as co-factor and a buffer containing ingredients and pH optimal for functioning of the polymerase (83–85). On the other hand, isothermal amplification only requires one temperature to amplify a target, using the same constituents to generate DNA strands plus proteins to separate the DNA strands (86). Both PCR and isothermal methods have drawbacks, notably related to requirement for equipment as well as sample complexity that impacts amplification.

Some considerations are important when doing molecular surveillance in samples. These include design of appropriate workflows (especially to prevent contamination of samples and thus generation of false samples). Importantly, quality, and trustworthy results are generated when molecular workflows are set up as per guidelines to prevent false outputs. These include unidirectional workflows, separation of processes and inclusion of appropriate controls (negative, positive, extraction and no template controls). The negative control is set up to ensure that there are no false positive results or contamination. The positive control is set up to detect amplification of the target fragment; while an internal amplification control (IAC) is set up, as a non-target DNA sequence, to detect false-negative results, particularly due to the presence of PCR inhibitors within the analysed sample. The IAC is co-amplified with the target sequence in the same tube as the sample. Other important considerations are discussed below:

a. Sample factors impacting amplification

PCR is sensitive to sub-optimal conditions and the presence of inhibitors in water and associated samples. These result in either weak amplification or completely restrict the generation of amplicons, resulting in false-negative reactions or delayed amplification (inhibition) (87,88). Known PCR inhibitors are $FeCl_3$, haemoglobin, fulvic acid, humic acid, tannic acids, bile salts, bilirubin, EDTA and NaCl (84,89).

These inhibitors may be introduced to the PCR in several ways, either during sample preparation, reagents used for nucleic acid extraction, or maybe residual, naturally occurring substances within the sample matrix. Examples of inhibitors found in sewage sludge are fats, proteins, polyphenols and heavy metals (90), whereas in wastewater common inhibitors are polysaccharides, metal ions and RNases (91,80,92). Humic acid found in soil and sediments also has inhibitory effects during PCR analysis (89,93). Inhibitors affect amplification by suppression of fluorescence (89). Polyphenol and urea are known to degrade DNA polymerases (91). Humic acid is a naturally occurring polymer formed from

organic material (degraded plants and animal tissues). Chlorine which is typically used to disinfect drinking water, is also known to have an inhibitory effect during PCR(94). Blood components (specifically haemoglobin, haematin) were previously shown to inhibit PCR through its impact on the functionality of the DNA polymerase most likely caused via iron trichloride containing compounds released from haemoglobin (e.g. iron chloride compounds) (95).

Dilution of water samples with high concentration of inhibitors are an option to limit the impact on PCR, but it also dilutes the actual targets. Numerous studies have been conducted using conventional PCR in different water environments, however, the method is limiting in that it only provides semi-quantitative results and is not sensitive with low amounts of amplicons (96,97). Rather than dilution of samples, it is more advantageous to remove inhibitors from samples. Various DNA extractions kits now include a step to remove inhibitors such as humic acid (98).

In light of the above, it is important to carefully select appropriate extraction methods and kits, to ensure optimal extraction of DNA that is inhibitor free and fit for downstream analysis (e.g. can be used for PCR or other methods such cloning).

b. Design factors impacting amplification

The design of primers and probes influences the reliability, sensitivity, specificity, and efficiency of the PCR reactions. Care should be taken to avoid non-specific amplification and generation of secondary structures such as primer-dimers and hairpins which can adversely affect the reaction. Various software is available to do these quality checks, and published guidelines enable quality checks of primers and rules to optimise real-time. PCR reagents (primer, dNTPs, magnesium, polymerase concentrations) need to be optimised, combined with checks for the optimal temperature to do specific amplification.

In qPCR, amplicon generation is monitored in real-time through fluorescently labelled probes or the incorporation of a fluorescent DNA binding dye in reaction mixtures; these fluorescent dyes are bound to newly synthesised DNA strands (99). qPCR is extensively used to detect and quantify microorganisms in environmental samples (100) and detect faecal contamination in water sources (101). The availability of dyes and real-time instruments that can detect it is another limiting factor as multiplexing to detect different targets is limited by the range of wavelengths that instruments can read. Generally, the more expensive instruments can detect most of the fluorescent dyes used in assays compared to cheaper instruments. Also, not all dyes are available globally.

c. Selection of appropriate amplification and detection methods

Several comparative studies using conventional, and qPCR have been conducted by research groups. Studies conducted by Staggemeier et al., (102) compared these two PCR approaches to detect Human Adenovirus (HAdv) in water and sediment samples (102). For qPCR, HAdv was detected in 87.3% of the water samples and 80% of the sediment samples, whereas it was only detected in 47.3% of the water samples and 35% of the sediment samples using conventional PCR. This indicates that real-time PCR is superior and more sensitive to conventional PCR. Another study conducted also used real-time PCR to detect and quantify emerging human and animal pathogens, namely *Aliarcobacter faecis* and *Aliarcobacter lanthieri*, in less than three hours from various environmental samples. This demonstrates the speed with which results can be obtained using real-time technology. In this study it was concluded that the qPCR assays developed could precisely measure the total quantity of *A. lanthieri* and *A. faecis* cells as well as their prevalence in complicated environmental samples (103). Yet culturing of these organisms required 3-6 days, using specialised media and microaerophilic conditions, thus counterproductive in terms of turnover time (103,104). This study is one of many that illustrate that qPCR assays are less time- and labour-intensive compared to culture-based methods, plus they are more sensitive to detect low amounts of template.

Unfortunately, as mentioned in Table 1.1, thermocyclers are expensive (in the context of poor resource environments), not only in terms of cost, but also maintenance (relevant for reliable results and laboratory accreditation so essential). Also, relevant technical expertise (trained staff) is essential to set up, validate and run molecular surveillance of (waterborne) pathogens (105). Thermal cyclers are essential for conventional PCR as these instruments are capable of temperature cycling, which is necessary for traditional PCR. These steps are used for activity of the heat stable Taq polymerase (generally at 72°C).

This limitation (of specialised instruments) initiated the development of isothermal DNA/RNA amplification (106), the process where DNA amplification occurs at a single temperature. Isothermal methods thus require a heated device set at one temperature and exclude the use of thermocyclers. Various isothermal amplification methods have been developed to date. Loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and Helicase dependent amplification (HDA) provide an alternative to the laborious culture-based methods (time consuming) and the use of specialised instrument-based PCR. These isothermal techniques have some drawbacks (mentioned in Table 1.2). The mechanism of action for these techniques are is represented in Fig. 1.4 to 1.6.

TABLE 1.1. INDICATING THE ADVANTAGES AND DISADVANTAGES OF CONVENTIONAL AND REAL-TIME PCR

PCR type	Advantages	Disadvantages
Conventional	✓ Easiest of the PCR types to perform. ✓ Low cost of equipment and supplies. ✓ Multiplexing of amplification of multiple targets in one reaction.	✓ Post amplification analyses are required, which increases time and labour as well as therisk of cross-contamination and human error. ✓ Amplicon products need to be confirmed sequencing. ✓ Unable to distinguish between live and dead cells, unless coupled with viability testing. ✓ High temperatures required for denaturing, annealing and elongation; need specialised instrument.
Real-time	✓ Less time-consuming compared to conventional PCR ✓ Less labour-intensive. ✓ Reduced chance of contamination due to automated detection ✓ Quantification range as low as 7–8 Log10 ✓ Multiplexing of amplification of multiple targets in one reaction.	✓ Unable to distinguish between live and dead cells. ✓ Requires more expensive equipment and reagents. ✓ Melt curves or expensive, fluorescently labelled probes needed to confirm identity of amplicons. Probe-based real-time more reliable. ✓ Need specialised instrument capable of temperature cycling plus fluorescent detectors.

Information obtained from (107), (108), (83), (109), (110)

Loop-mediated Isothermal amplification (LAMP)

LAMP technology, developed by Notomi et al., (2000), amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions (111). It is performed at a constant temperature, thus enabling amplification using a water bath or heating block. Detection is simple, both synthesised amplicons and a white precipitate (the pyrophosphate by-products increase the turbidity of the solution) can be detected (112,113). LAMP requires two sets of forward and reverse primers, inner and outer primers, and a DNA polymerase with strand displacement activity (Fig. 1.4). LAMP assays have been developed for various pathogens (114).

Contrary to Notomi's claim of high specificity, some studies observed non-specific amplification using LAMP. For example, one study compared the performance of LAMP, conventional PCR, nested PCR, and real-time PCR using *Entamoeba histolytica* DNA derived from faecal samples. Despite the popularity of these amplification methods in disease diagnostics, the high DNA similarity amongst species and low copy number of specific genes for pathogen identification are major drawbacks resulting in false positives due to amplification of non-targeted, closely related species (115). Another study using LAMP for simple and rapid detection of clinically important urinary pathogens found high specificity (100%) for LAMP assays targeting selected bacteria; more importantly, it also noted that the sensitivity of the assays was around 1.5 × 103 cfu/ml, which is the cut-off value used to define bacteriuria or UTIs in patients with symptoms (116). Non-specific amplification via LAMP is due to technical limitations such as low temperature and primer design (117). Using additives such as ammonium sulphate, tetramethylammonium chloride (TMA), acetamide, betaine, dimethyl sulfoxide, formamide and non-ionic detergents inhibit non-specific amplification in a concentration-dependent manner (meaning it likely has to be optimised for different assays and targets) (118,119). Thus, LAMP is generally highly specific, there can be instances of non-specific amplification due to various factors (such as low amplification temperature, poor primer design and others as mentioned earlier) and further optimization (such as the addition of the abovementioned chemicals) may be needed to enhance its specificity.

Another method to improve specificity and sensitivity, is to use a self-avoiding molecular recognition system (SAMRS). SAMRS refers to the use of modified nucleotides in the primers. These modified bases can bind to their normal (non-modified) nucleotide complement but not to a modified complement (e.g. modified G can only bind to non-modified C). Thus, if the primer nucleotides (e.g., all the G and Cs) in primers are all modified, the primers cannot bind to one another, only to the template DNA (120,121).

TABLE 1.2. DRAWBACKS OF THREE COMMONLY USED ISOTHERMAL METHODS AND SUGGESTED SOLUTIONS.

PROBLEM	MECHANISM	SUGGESTED SOLUTION
Non-specific amplification	The amplification temperature below standard 72°C results in risk of primers binding off-target. LAMP use more than 2 primers, potentially all contributing to off-target amplification RPA prone to non-specific amplification as reactions shown to occur even at room temperature	Use self-avoiding molecular recognition system (SAMRS), or compounds such as TMA Primers must be extremely well designed, plus all quality checks done to ensure optimal, specific amplification. These include titrations to discern optimal concentration of reagents, well designed primers that can be used at minimal amounts. For RPA, ensure to mix in template and sodium acetate in last (directly before putting in heated device).
Non-specific detection	Off target amplification and primer dimers also visible in tubes if fluorescence is measured or detected by eye. Turbidity in LAMP reactions not reliable indicator as it can be caused by both specific and non-specific amplification and primer-dimer formation.	Confirm correct amplification on gels. Use probes instead of SYBR for fluorescent detection. Use probes (these are highly sequence specific) instead of SYBR-I dye (bind to any double stranded DNA, INCLUDING primer dimers) for fluorescent detection. Use turbidity plus an additional marker to confirm correct amplification
Cross contamination	High number of amplicons formed (more than in PCR amplification), very easy to contaminate environment (gloves, etc.).	Maintain unidirectional flow. Potentially change gloves in-between opening different tubes, especially if products are used for downstream cloning or hybridisation experiments.

Information obtained from (122,123,112,124–129).

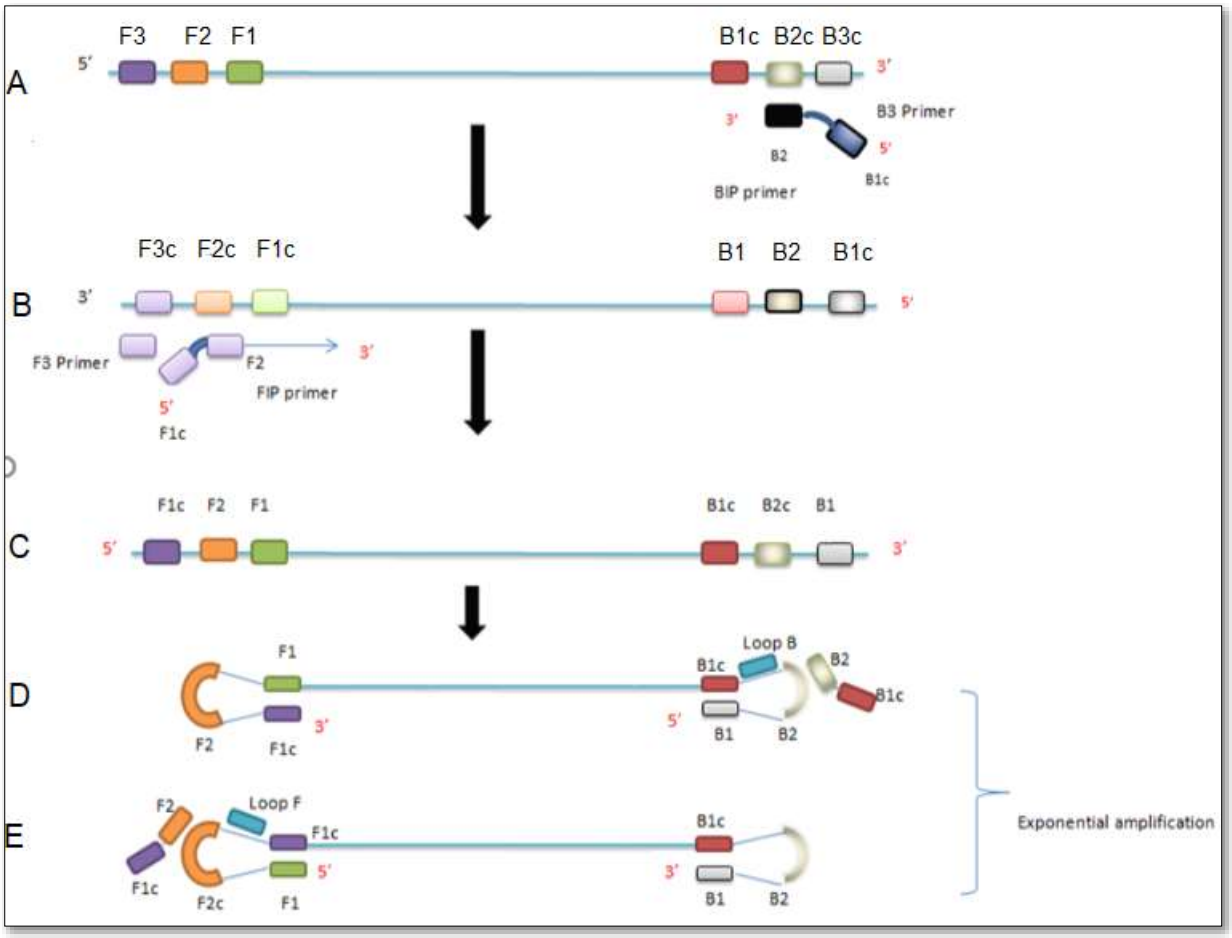

Fig. 1.4. Principles of amplification via LAMP

LAMP reactions consist of dNTP mix, *Bst* polymerase, primers and DNA template. The *Bst* polymerase functions at a lower temperature (60-65°C) than extremely heat stable polymerases used in typical PCR reactions. **A**: The backward inner primer (BIP) consists of 2 sequences: the (B2) region on the 3'end of the reverse BIP primer is complementary to the B2c region of the template. The 5' end of the primer (B1c) is homologous to the sequence just upstream of B2 that is the B sequence. **B**: The forward Inner Primer (FIP) consists of a sequence homologous to the F2 region (3'-end) and a sequence just downstream of it (F1) in the 5'-end that is homologous to the F1c region of the template (112).

The F3 Forward outer region of the primer complements the F3c region of the template sequence. C: Complementary strand synthesis is initiated by the FIP region's hybridization to the target DNA's F2c region, and this is followed by the F3 primer's extension and hybridization to the target DNA's complementary F3c region, which displaces the complementary strand linked to the FIP. D: The displaced strand creates a loop with a 5' end that acts as a model for BIP. The template DNA's B2c region is where B2 hybridizes. The initiation of DNA synthesis results in the production of a complementary strand and the opening of 5'-end loops. The BIP-linked complementary strand is then displaced as B3

expands and hybridizes with the target DNA's B3c region. As a result, DNA in the shape of a dumbbell is created. Bst DNA polymerase adds nucleotides to the 3'-end of F1 by 5´ → 3´ polymerase and 5´ → 3´ exonuclease activity, which stretches and opens the loop at the 5'-end. Section D–E: The DNA in the form of a dumbbell is then changed into a stem-loop structure. The dumbbell structure, the second stage of the LAMP response, starts the LAMP cycling. Additionally, loop primers can be added to LAMP for exponential amplification. The resultant products are a mixture of multiple-looped cauliflower-like structures and stem-loop DNA with varying stem lengths. (112,130).

The use of multiple primers, as done in LAMP, has multiple drawbacks. This, together with some solutions and its functions are indicated in Table 1.2.

A major feature of LAMP is the number of amplicons formed during amplification and thus potential for cross contamination. One study (123) noted that observed cross-contamination of samples may have been due to the opening of the tubes to add the SYBR green dye post amplification, suggesting post amplification steps must be limited. This contamination (due to opening of tubes to add the fluorescent dye post-amplification) had also been reported by others (131).

Despite some limitations LAMP had been used for the detection of *Giardia* successfully in one study (132), outperforming all other techniques in terms of robustness, cost-effectiveness, high specificity, and sensitivity. This study assessed the efficacy of three detection methods (Immunofluorescence Test (IFT), PCR (temperature cycling based amplification) and LAMP and used DNA from several water types (surface waters, groundwater, untreated water from a drinking water plant, recreational area, effluent, and wastewater treatment plants (WWTPs) to detect the microorganism. IFT fared better than LAMP and nested PCR (56.8 > 42.7 > 33.5%, respectively). The comparison of the three methods indicated better results with IFT compared to the DNA detection assays, and the LAMP assay was more sensitive than the PCR method for the detection of *Giardia* (132). However, a major limitation to using IFT is that cross-reactions with closely related antigens, may occur, it may be expensive and time-consuming and false positive results can occasionally arise from antibodies binding non-specifically to unrelated molecules (133). In this regard, DNA detection might be a more feasible option.

Recombinase polymerase amplification (RPA)

Recombinase polymerase amplification was developed in 2006 (134). Research conducted by Lillis et al.,(135), indicated that RPA can work at ambient temperatures in warm areas

above 30°C (135) though more recent work showed effective amplification at room temperature and body heat (136,137). A schematic diagram below (Fig. 1.5), indicates how RPA works.

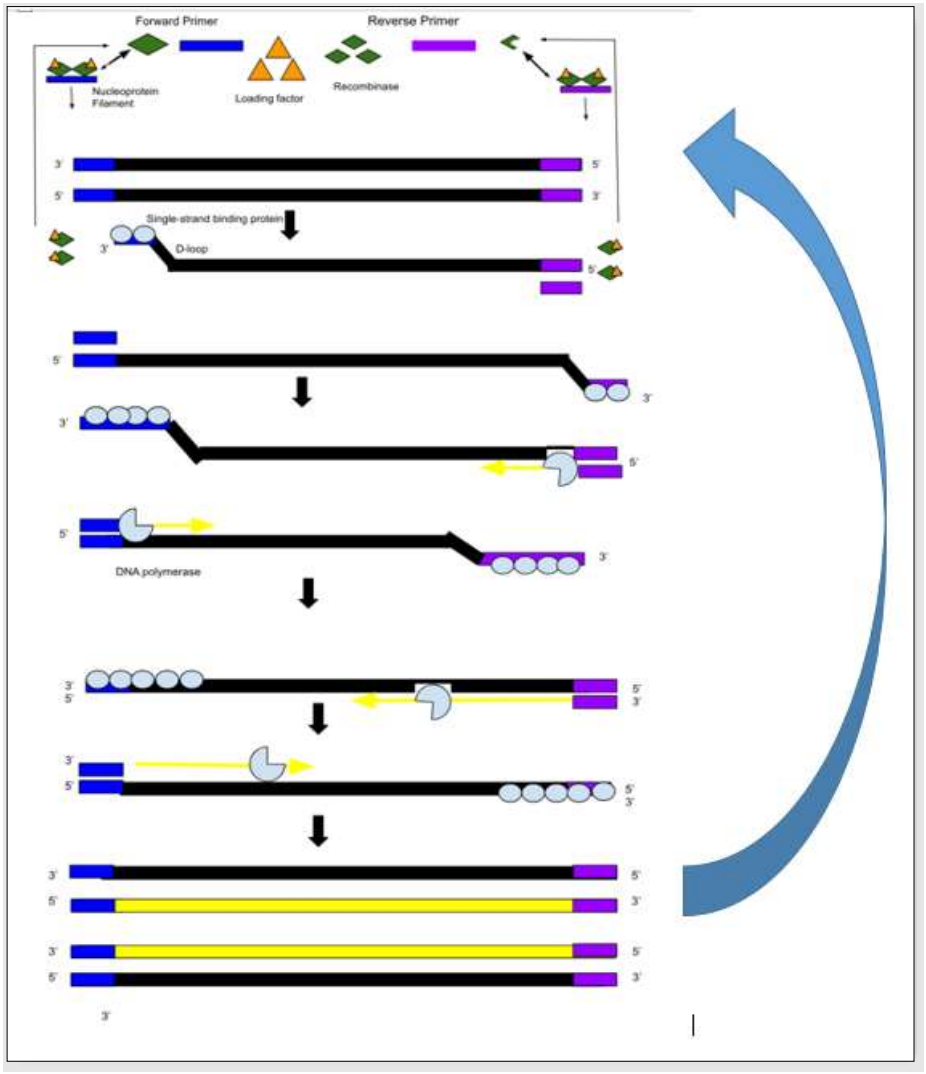

Fig 1.5. Diagram of the RPA analysis process

A loading factor (phage *T4* recombination mediator protein, T4 UvsY) assists a recombinase (T4 UvsX) to bind to the primers to initiate the RPA reaction. The resultant nucleoprotein filaments search the double-stranded DNA for homology (identical sequence) to primer sequences. After finding the correct sequence, the complex enters the duplex DNA (strand invasion), leading to the formation of a D-loop structure. Single-stranded binding protein (T4 gp32) stabilies the unwinding strand by binding to ssDNA and preventing duplex formation throughout this process. A polymerase (such as *B.subtilis* Bsu) uses provided nucleotides to generate new DNA strands, formed from the 5' towards the 3' direction. The result is two newly formed two duplexes from a double stranded template. This process is repeated until reagents are depleted, and the products can be viewed post-amplification via UV fluorescence or real-time monitoring of labelled amplicons using fluorescent dye incorporation during the amplification process. Figure courtesy Li et al., 2019 (138)

To date, RPA to detect various pathogens has been developed, including viruses (139–142), protozoans (143), bacteria (144), fungi (145,146) and parasites (147) (Table 1.3). With regards to parasites, it is a noteworthy step, as there is a need for improved and rapid detection of parasite eggs in soil and water (148). RPA assays to detect parasites, such as those developed by Ravindran et al. (2020), thus are an important stride to do fast and cost effective surveillance of water close to communities that might expose such communities to parasite eggs. The abovementioned study reported on an RPA assay, coupled with a lateral flow strip, to detect helminth ova in wastewater. This multiplex assay was able to successfully detect DNA from ova from two Helminth genera (*A. suum* and *T. suis)* in a single lateral flow strip. To test the specificity of the primers, DNA from *A. suum* and *T. suis* was used, with other animal helminths (*Trichuris suis* and *Haemonchus contortus*) as test pathogens for specificity. The study concluded that the RPA-LF strip successfully detected the helminth ova DNA at levels as low as 2 femtogram. Importantly, amplification occurred using the body temperature of the investigators, thus excluding even the need for a heated device (149). As stated previously, other investigators also showed effective amplification using body heat (150).

A significant feature of RPA is sensitivity, enabling the detection of very low numbers of targets. For example, studies conducted by Saxena et al,. (2019) successfully detected *B. mallei* in artificially spiked water and garden soil, detecting DNA from little as 2.1×10^3 CFU of *B. mallei* per ml of artificially spiked blood. Other studies also reported better sensitivity (151). Detection of the waterborne pathogen *L. pneumophila* was more sensitive, with 1.6×10^1 colonies/ml of water being detected via RPA (152). This was comparable to the results obtained using real-time PCR in same report. In the case of the fluke *Fasciola hepatica*, as little as 1.6pg of DNA purified from stools was successfully detected (153). In the case of SARS-COV2, sensitivity as low as 7.6 copies of RNA were reported to be detected in patient samples (154). Studies conducted using the RPA-LFD test demonstrated a sensitivity of 100 times greater than RPA for the detection of recombinant plasmid DNA (1.9 × 100 pg/µl) and a sensitivity of 10 times greater than RPA for the detection of genomic DNA (2.0×10^2 pg/µl) (155). This observation is interesting, as it implies that targeting plasmids (which multi- copied) instead of genomic DNA (which would be one or a few copies depending on the ploidy) would improve the sensitivity of the amplification assay.

RPA is a rapid solution for the diagnostic detection of pathogens. For example, an assay had been developed to detect bacterial contamination in plasma products. If incorporated into diagnostic settings or blood bank laboratories, it could assist with the quality

management of blood products (156).

However, RPA analyses are not devoid of drawbacks as indicated in Table 1.2. These drawbacks can be addressed in ongoing development as the rapid turnover time and ease of use of this method (especially when combined with lateral flow assays) holds real promise for point of care (POC) detection to assist diagnostics and quality control of food, medicines, water and other products meant for human consumption.

TABLE 1.3. RPA DETECTION OF MICROBES REPORTED IN THE LITERATURE

Microbe	Amplicon Detection method (RT/LFA/GELS)
Bacteria	
Streptococcus	RPA coupled with Lateral flow dipstick
L. pneumophila	EuNPs-based lateral flow immunochromatography
L. pneumophila	Dual labelled amplicons detected on lateral flow dipsticks
S. pneumonia	Dual labelled amplicons detected on lateral flow dipsticks
Fungi	
Phytophthora	RPA coupled with Taq-man amplification
Bipolaris sorokiniana	RPA coupled with fluorescent detection
Large parasites and protozoans	
Schistosoma haematobium	Real time fluorescence-based RPA
Cryptosporidium	RPA coupled with Lateral flow dipstick
Viruses	
SARS-CoV-2	Reverse transcriptase RPA coupled LFA and fluorescent detection
Human Immunodeficiency Virus	RPA coupled with Lateral flow dipstick and fluorescent detection `
Rift Valley fever	Reverse transcriptase RPA
Monkeypox	RPA coupled with transcription LFA together with self-design qPCR
Monkeypox	RPA coupled with transcription and fluorescent detection of CRISPR cleaved products

Information obtained from: (139,136,140–142,145,152,157–160).

Helicase-dependent amplification (HDA)

Helicase-dependent amplification was developed in 2004 (161) but unlike PCR, HDA uses helicase enzymes to separate DNA duplexes and do not require elevated temperatures (94-95°C as used in PCR) to obtain single-stranded DNA (162–164). Helicase enzymes are used in all living cells during DNA replication, repair and recombination. It separates double-stranded DNA so that the single strands can serve as templates for reactions (such as the building of new DNA strands) to occur (165). This ability, to break the double-stranded bond of DNA at temperatures relevant to living cells to survive, is utilised for DNA amplification via HDA, as illustrated in Fig. 1.6.

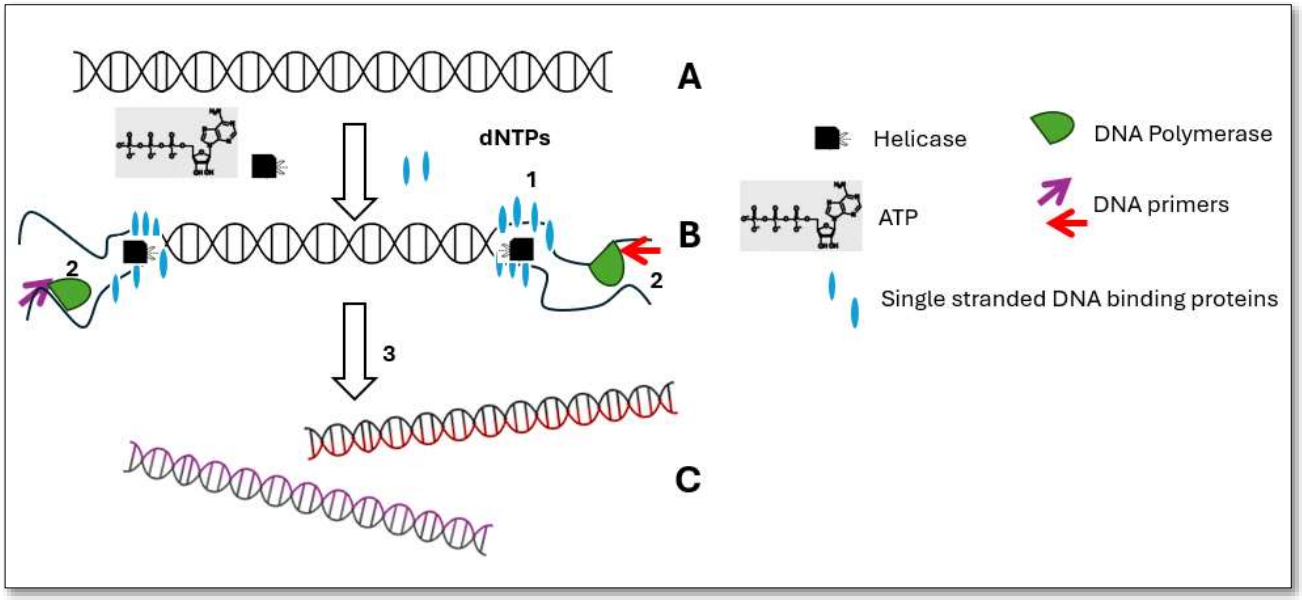

Fig. 1.6. Diagram of the HDA analysis process

A. Double-stranded DNA is unwound in the presence of a helicase, ATP, and DNA binding proteins.
B. A helicase denatures the dsDNA, thereafter, single-stranded binding proteins bound to the single strands ensure the double-stranded structure does not form again. Primers hybridize to with their homologous counterpart on the template, followed by the synthesis of a new complementary strand of DNA. The new double-stranded amplicons serve as templates for each new round of amplification. The process whereby helicase unwinds DNA is , enzymatic with amplification thus requiring only a single temperature throughout the process (160).

HDA assays for pathogens had been described in the published literature (166–168), though limited peer reviewed publications could be found where this technology was used. Studies conducted by Kolm et al., (163) lead to the development of an *Enterococcus* HDA assay which successfully discriminated between 15 enterococci and 15 non-enterococci reference strains and detected 48 environmental enterococci isolates (163). Vincent et al., (161) indicated that the simplicity and use of a constant temperature in HDA-technology, could be

very appealing in developing handheld point of care (POC) DNA diagnostic devices to detect pathogens in the field (127,161). An advantage of HDA over LAMP is that the reaction setup is quite simple, and its PCR-like reaction scheme allows for the adaptation of the current PCR assays to the HDA system. Studies conducted by Kolm et al., (163) successfully developed a HDA strip assay for identifying faecal pollution sources in water by targeting one of the most prominent microbial source tracking markers, BacR (163). Once the limiting primer is exhausted, the procedure, which is carried out wholly on a conventional heating block, yields single-stranded BacR copies. It is based on the employment of one primer in excess concentration. Next, by immediately pouring the final HDA mixture onto the sample application pad of the test strips, the amplification products are detected using the nucleic acid lateral-flow strip test. In terms of sensitivity, HDA were found to be more sensitive, compared to LAMP, as a technique to detect SARS-CoV-2 (3 genome copies per reaction within 20 minutes vs 30–300 genome copies per reaction within for 40 min) (168). Despite these successes, HDA technology still needs to overcome some important limitations such as the inability to distinguish between viable and non-viable cells (169), a common issue for all nucleic acid amplification techniques. Methods to overcome this limitation, i.e. to distinguish between viable and non-viable cells, will now be discussed.

Limitations of DNA detection methods and solutions

Amplification of nucleic acids is not proof that viable microbes are currently present in a system. It is known that intact DNA may persist from a few days to three weeks following cell death. Therefore, amplification results should be interpreted carefully as the presence of living microorganisms may be overestimated (170) .

To distinguish between viable and non-viable cells, photoreactive, DNA binding dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) are used. Living cells with intact cell envelopes prevent/limit dye entry, while dyes enter injured or dead bacterial cells via the compromised membranes and bind to nucleic acids inside the dead cells. These dyes also bind cell-free DNA in the external environment (110). Amplification cannot be done using these dye-DNA complexes. Therefore only unbound DNA obtained from viable cells are detected and amplified (75,171). These dyes can be included before DNA extractions to form dye-DNA complexes derived from dead cells, or in amplification mixtures when using crude DNA (96). An example of this application was reported in a study where PMA was used to distinguish between live and dead bacteria was the detection of *Pseudomonas* in swimming pool water (172). A recent study conducted by Yuan et al., (171), using PMA, was able to successfully detect 10^3 cfu/100 mL viable *E. coli* cells in the presence of

105 cfu/100 ml of dead cells (171).

However, the drawback of this method is that viability is based only on membrane integrity. Another limitation of the use of PMA is that the cells must be used in light-transparent solutions (e.g., water samples, cell cultures, etc.) as the intercalation of this photoreactive dye to DNA requires exposure to light. Samples of insufficient light transparency do not permit the application of these dyes (110,173). Studies conducted by Li et al., 2014 indicate that certain sample matrices, such as sludges, may inhibit the efficacy of PMA limiting the differentiation between viable and non-viable cells within the sample due to the presence of dark particles in sludge (174).

In addition, disinfection methods such as UV-radiation damages the DNA in the bacterial cell, for example UV-induced thymine dimerization inhibits DNA amplification. As the cell membrane is still intact, the PMA treatment may be ineffective in detecting the DNA from the non-viable cells (169,175).

Other DNA intercalating dyes are also available (EMA), but not all are equally effective. Comparative studies on PMA versus EMA to discern *E. coli* 0157:H7 and *Campylobacter* viability in broth cultures indicated that EMA penetrated the membranes of live bacterial cells (176–178). Additionally, it caused about 60% of the genomic DNA of the live cells that were taken during the log phase to be lost. The findings also suggested that the bacterial species and the molecule's larger charge—PMA has two positive charges compared to EMA's one—were likely responsible for PMA's noticeably higher selectivity. (176).

The usefulness of the abovementioned isothermal methods increases when detection is also rapid and requires minimal instrumentation, such as offered by point of care devices.

Point of care devices

Another important aspect of rapid testing is the detection method of the generated amplicons. These can be cumbersome (such as UV fluorescent DNA on agarose gels or expensive (such as fluorescence detection of labelled amplicons via using real-time PCR). These methods require specialised instruments and stable electrical capacity. A trustworthy and steady electrical supply is essential to preserve equipment life and reliable PCR results. Therefore, especially in resource poor settings (that frequently lack electrical supply or have poor infrastructure), the use of methods requiring no electricity is more feasible. Note that stable electrical infrastructure is a requirement for reliable and quality delivery of diagnostic services (https://extranet.who.int/lqsi/content/ensure-uninterrupted-supply-electricity). More so, POC testing is more feasible in remote settings with limited access to laboratories,

especially with water samples that must be processed within specified time constraints for quality outputs and compliance (179–181). The ideal POC device would be portable, easy to use, requires minimal or no infrastructure and require minimal training to operate the instrument. Such devices ideally facilitate both amplification and detection of amplicons or amplification on POC instruments with detection via methods requiring minimal/no electricity. Detection methods for amplicons range from agarose gel electrophoresis which can be done in a mobile field-in-a-case laboratory), via POC real-time PCR detection devices, or through paper-based strips that capture and visualise labelled amplicons that can be seen with the naked eye. An example of the latter is lateral flow detection devices.

Lateral flow strip assays (LFA, also referred to as immunochromatographic assays, dip or strip assays) are increasingly popular in diagnostic settings as they provide results within minutes of applying a sample to the device and require no electricity (182,183). LFAs visualise antigen/antibody-labelled targets on a test line where an appropriate antibody/antigen is immobilised and thus the target is concentrated in that line. Combined with chromogenic or fluorophore detection, the concentrated, captured target is visible either with the naked eye or via light at a specific wavelength. First introduced as a urine-based pregnancy test in 1984 (183), LFA-based tests are now commonly used in clinical laboratories and hospitals for both quantitative and qualitative detection of specific antigens, food safety assessments, quality control and environmental health (183,184).

An LFA device typically comprises of four segments: a sample pad, a conjugate pad where test molecules interact with, a testing/detection zone (membrane) where the interacting molecules are captured and concentrated, and lastly, an absorbent pad (183,185,186). Segments are made up of different porous material, overlapping one another and pasted to a backing card by means of pressure-sensitive adhesive. These materials are fused silica, ether nitrocellulose, polyethersulfone, nylon, or polyethylene. Ligands (antibodies, oligonucleotide or aptamers) are placed at pre-determined locations over the capture zone, where it functions to capture specific targets if these are present (185).

The sample pad, the area to which the sample is added, is saturated with buffers containing salts or surfactants. These chemicals facilitate the dispersal of amplicons across the conjugate pad and test strip (183). The conjugate pad contains antibodies or aptamers which are specific to analytes and then conjugated to coloured particles, gold nanoparticles or latex beads (186). The sample (e.g. labelled amplicons) migrates through the conjugate pad containing the antibodies which is specific for the target amplicons, then conjugates to the nanoparticles or beads (184). Thereafter, this complex (amplicon-conjugated antibody-

target chemical analyte) migrates along the strip to the detection zone where antibodies or antigens capture the complex in the detection lines (183,184,186). Once the sample analyte (for example a labelled amplicon) is recognised (in the detection zone),a response is noted on the test line in the form of a colorimetric (such as streptavidin-biotin interaction) or fluorescence signal (such as SYBR-1 or fluorescein isothiocyanate (FITC) (183). The last segment on the strip is the absorbent pad which captures excess liquid and any unbound amplicons (184). An additional function of the absorbent pad is to hold the liquid for the duration of the assay (185).

LFAs are categorised into different types based on the recognition elements used and the type of analyte to be identified. When the test strip is designed with antibodies as its recognition element, it is called a lateral flow immunoassay (LFIA). There are two formats of LFIA, namely the competitive assay (indirect form) and the sandwich from assay (direct form) (183,185,187). The competitive assay is used when testing for smaller molecular weight analytes with single antigenic determinants that are unable to bind to two antibodies at the same time (186). With this format the positive result is indicated by the absence of a test line. When the result is negative both the control and test lines will appear. In the case of the sandwich format, it is used when the molecular weight analyte is large with multiple antigen sites. The positive result is indicated by the concurrent display of test and control lines whilst a negative result is only displayed by the control line (187).

Advantages and Disadvantages of LFAs

The advantages of LFA strips are that requires they are inexpensive, can be used by an unskilled person, are highly sensitive and specific, and require no refrigeration (184,185,187). However, there are some drawbacks such as the inter-individual subjectivity associated with visual interpretation (185). Also sample pre-treatment is required for non-fluid samples (such as food particles). Incorrect sample volumes can reduce precision. Also, matrix compounds (such as those found in crude DNA preparation) may cause blockages in the pores of the strip (184). In this regard, purified DNA used for PCR would be more advantageous as opposed to crude DNA (not purified, but sample merely boiled, thus containing any non-DNA material present in the sample). Therefore, extracting DNA from samples would be an important step in the use of LFA strips. The last section of this review will focus on DNA purification as an important step in molecular surveillance.

DNA extraction methodologies

Lysis, precipitation, and purification are the three processes required for DNA isolation. A typical DNA workflow consists of rupturing the cell and nucleus to liberate the DNA (lysis), followed by DNA purification (the process of washing the DNA with a chemical agent, for example alcohol to get rid of undesirable cellular debris) (188). Due to different microbial species having different cell wall structures and being more/less susceptible to different lysis procedures, variations in extraction methodology can result in varied concentration and purity of extracted DNA (189).

Different chemicals are used to lyse bacterial cells and remove unwanted biological residues (proteins, carbohydrates) via digestion of proteins, precipitation, and purification (different methodologies employed). Extractions can be done using in-house prepared chemicals or via various commercial kits available (190). Kits in general, employ solid matrices or magnetic bead to separate nucleic acids from cellular debris and purify nucleic acids. For example, the Mag-bind ® DNA 96 kit (Omega Biotek, USA) uses Mag-BIND® particles to reversibly bind DNA or RNA allowing proteins and other impurities to be eliminated. Firstly, bacterial cell walls are broken using lysozyme digestion and Proteinase K digestion combined with a chaotropic reagent[1] (e.g. guanidine thiocyanate). Once the sample has been lysed, the binding conditions are modified via changes in the buffer salts and pH, magnetic beads are added to the mixture to bind the DNA (Mag-Bind® Bacterial DNA 96 Kit | Omega Biotek (omegabiotek.com). Magnetising the DNA-magnetic bead conjugate separates it from the liquids that can be removed. Repetition of this step with wash buffers purifies the nucleic acids before it is finally separated from beads in an elution solution. Other kits use solid matrices packed in columns, where DNA will bind, washed repeatedly with a set of buffers, then eluted also by changing to a buffer that will elute the nucleic acids off the packed in columns. Examples of commercial kits in use for water surveillance are the FastDNA® Spin Kit for Soil (MP Biomedicals), PowerSoil® DNA Isolation Kit (Qiagen) and ZR Fecal DNA Miniprep kit (Zymo) are other popular, spin column- based kits used in various reported studies on wastewater and freshwater sediments (191,192). Another popular kit is the magnetic bead kits such as Magna Pure 96 and MagnaExtract (193–196). Where laboratories can afford it, automated extractions are also done, but manual kits are more practical to be used in the field, especially magnetic bead kits that do not require electricity for most steps (except vortex and initial centrifugations steps if needed).

[1] Chaotropic reagents interfere with hydrogen bonds in biological molecules, assisting with cell lysis

As stated previously, the ideal POC testing would be done using devices that are portable, use minimal electrical power and either a closed system (extraction and amplification in one tube or with extraction in one section/tube and amplification in another section (and sections linked to one another to prevent additional external manipulation and pipetting). In this regard, there are examples of POC prototypes reported in the peer reviewed literature, such as the POCKET (point-of-care kit for the entire test) ultraportable device (less than 100g in total, shorter than 25cm, fitting in a bag with dimensions of 17 cm × 25 cm) (197). Consisting of a chip (where DNA is absorbed and extracted from a sample) and a smartphone to generate heat for amplification plus to do signal detection of amplicons (thus a "reader"), it was demonstrated to successfully extract and detect below 10^3 copies/ml of DNA targets from clinical, food and river water samples. Some components of the device were 3D printed. Another POC device, using RPA and various modes of detection, described in the literature is the POC-CRISPR (using RPA combined with CRISPR-Cas) in a fully integrated device for nucleic acid preparation and target amplification plus detection (198).

Analysis of *L. pneumophila* and *C. perfringens* in water samples

According to the standardised guideline (Methods such as ISO 11731 Water quality - Enumeration of *Legionella*), an incubation period of 7-10 days is required to culture *Legionella*. Thereafter, presumptive colonies must be confirmed which requires an additional 2-5 days. For the enumeration of *C. perfringens* (vegetative cells and spores), the ISO 14189 standard requires an analysis period of 21 hours (±3hrs) with an additional 21 hours (±3hrs) for the confirmation of presumptive colonies. After the incubation period is complete the acid phosphatase test should be performed. Detection of *C. perfringens* is confirmed by the development of a purple colour. Rapid testing will improve on current culture-based methods, which are laborious and lengthy to detect and confirm the pathogen's presence. The abovementioned procedures thus are lengthy and delays time to result, which in turn impacts follow up procedures (such as public health responses if samples contain relevant pathogens). Therefore, rapid technologies would hugely impact the time-to-detection of pathogens in water.

Rapid technologies for the detection of *C. perfringens* in water include DNA microarray (where multiple pathogens are detected) (199) and real-time PCR (200). Rapid technologies for detecting *L. pneumophila* include biochemical testing and immune detection methods (152,201–203). For example, the Legipid® Legionella Fast Detection test is based on combined magnetic immunocapture and enzyme-immunoassay. This rapid test utilizes immunomagnetic separation by anti-Legionella immuno-modified magnetic beads combined

with an enzyme-linked colourimetric detection (203). Another commercial kit for rapid *Legionella* detection, is the LegionellaFast, which detects the presence of *Legionella pneumophila* serogroup 1 using an LFICA (Lateral Flow Immunochromatographic Assay) (204).

Considering the abovementioned discussion, this study aimed to evaluate an isothermal DNA amplification method that would potentially minimize the use of electrified equipment and decrease turnover time to detect targeted pathogens. We used two resilient microorganisms, namely *C. perfringens* and *L. pneumophila* which are known to withstand traditional chemical treatment inactivation methods. Towards this, primers were designed and optimised to develop a rapid molecular method to detect *C. perfringens* DNA using recombinase polymerase amplification and a commercial lateral flow device. In addition, we used a previously described primer set to detect *L. pneumophila* DNA using Recombinase Polymerase Amplification (RPA), validating that the method works in our study. RPA generated amplicons were detected via lateral flow on a commercial LFD device. Furthermore, the RPA-LFD method was validated by testing a limited number of water samples from various matrices. Lastly, as DNA extractions are a crucial step towards generating DNA from different water matrices, we also evaluated three commercial DNA kits that are locally available and used three different modes of DNA purification, i.e., either a solid-phase column DNA clean-up step, a magnetic bead purification method, or a chemical clean-up method.

In summary, molecular methods provide an opportunity for improved and more rapid detection of pathogens in systems such as water. Limitations to molecular technology include the cost of reagents and equipment, electrical requirements for traditional molecular analysis, availability of an appropriate cold chain to store reagents, access to reagents as well as the level of technical skill required. Additionally, outcomes of nucleic acid amplification assays may be incorrectly interpreted and are not evidence of live microbes in a system (this can be overcome by coupling PCR with live-dead detection) (176). In addition, molecular assays are susceptible to contamination, but this can be overcome by taking precautionary measures). Molecular methods appear to be a more feasible option to detect microorganisms rapidly. Point of care devices can further assist by allowing analysis at the site of suspected contamination of water. However, there should be consideration for appropriate DNA extraction methods and different water matrices should be considered, and methods effectively optimised for different matrices, cost, and ease of use for reliable results.

AIMS AND OBJECTIVES

The aim(s) of this project was:

To develop and optimize a rapid molecular method to detect selected microbial pathogens, *C. perfringens* and *L. pneumophila*, using recombinase polymerase amplification and a commercial lateral flow device.

The objective(s) were:

1) To optimize RPA conditions to amplify the phospholipase gene of *C. perfringens* and the *L. pneumophila* Serogroup I - specific *mipA* gene, using pure genomic DNA.
2) To optimize the method using different types of water (drinking water, pre- and post-sewage treatment effluents, marine outfalls, re-use, river, swimming pool, tidal pool and industrial wastewater in Cape Town monitored by the local authority), to improve sensitivity across different specimen types.
3) To optimize the detection of PCR products using a commercially available LFA strip.

Chapter 2 Materials and Methods

2.1 Bacteria, samples, media and growth conditions

Lyophilized bacterial strains of *C. perfringens* ATCC® 12915™ Kwik-stik™ (Microbiologics, USA catalogue No. 0801P) and *L. pneumophila* ATCC® 33152™ Kwik-stik™ (Microbiologics, USA catalogue No. 0211P) were purchased. The Kwik-stiks™ were reconstituted as per manufacturer instructions. These reconstituted bacterial materials were inoculated onto the appropriate culture plates and streaked to obtain single colonies.

C. perfringens was cultured on brain heart infusion (BHI) agar (Bacto™, Thermo Fisher Scientific, USA) or 2 % blood agar plates (horse blood, Brucella base, prepared by Green Point Media, South Africa). The plates were incubated anaerobically at 37 °C for 24 hours in an anaerobic jar containing a sachet of AnaeroPack® (Mitsubishi Gas Chemical, Japan).

For *Legionella*, charcoal yeast extract agar (CYEA) (Oxoid, CM0655 Thermo Fisher Scientific, USA) was supplemented with *Legionella* buffered charcoal yeast extract (BCYE) growth supplement, (SR0110 Thermo Fisher Scientific, USA) and GVPC (Oxoid SR0152 Thermo Fisher Scientific, USA) to form Buffered Charcoal yeast extract agar. *L. pneumophila* was cultured on this medium and incubated aerobically at 37°C for 72 hours. Culture purity, as well as colony and cell morphology and correct identity of the target microorganisms were confirmed using visual inspection of colonies and the Gram staining technique. Typical colony and cell phenotypic features of both microorganisms were accepted as preliminary confirmation of identity (prior to the sequence of DNA amplicons to confirm identity).

Water samples to validate the RPA method were obtained from the Scientific Services Branch, City of Cape Town Municipality. Samples were de-identified regarding the specific names of the rivers, dams or sewage plants.

2.2 Development of molecular detection method for *C. perfringens plc* gene.

To develop the method, DNA was extracted from a reference strain, three commercial kits were also evaluated for the ability to obtain fit-for-purpose. DNA from cultures of the organism, thereafter an RPA method to amplify the *plc* gene was optimised, amplicons detected via agarose gels and a lateral flow device. To verify that we used the TwistDx RPA technology successfully, we also evaluated a published method to detect the *L. pneumophila mipA* gene and detected amplicons via agarose gel electrophoresis and a lateral flow device.

2.2.1 gDNA extractions

Initial DNA extractions, to optimize PCR conditions and obtain amplicons to sequence, were done using the Quick-DNA Fungal/Bacteria Miniprep kit (Zymo Research, USA, protocol: Quick-DNA™ Fungal/Bacterial Miniprep Kit (zymoresearch.com)). For this, a loopful of bacterial cells were scraped off plate cultures, and washed in 1 ml PBS once (centrifuged at maximum speed, 1 minute (Prism Mini Micro centrifuge Labnet, Edison, New Jersey, USA). Hereafter all supernatant PBS, except about 200 µl and the cell pellet, were discarded while the pellet was re-suspended in the remaining PBS. DNA extractions from *C. perfringens* and *L. pneumophila* were done in triplicate according to the manufacturer's protocols, except that DNA was finally eluted in 50 µl of elution buffer (instead of the advised 100 µl). This kit includes a mechanical cell disruption step (hereafter referred to as "beat eating"), done using the Disruptor Genie (Scientific Industries, USA); this step was done for 5 minutes. DNA quantitation was done using the Bio-drop Duo spectrophotometer (Biochrom, UK) and NanoDrop™ 2000 Instrument (Thermofisher, USA). The isolated gDNA was stored at -20˚C until further use in PCRs to confirm identity as well as RPA test development. Following this, all further DNA extractions were done as described in Section 2.2.2 (where genomic extraction using different commercial methods were described).

2.2.2 Comparison of DNA extraction kits.

To compare the efficiency of three, locally available DNA extraction kits, representing three different modes of DNA purification, were selected to extract DNA from pure, cultured *C. perfringens* cells. DNA was extracted from equivalent amounts of bacterial cells, prepared from the same culture, and standardised using McFarland standards. For this *C. perfringens* were cultured as described in 2.1.1, cells harvested in PBS and diluted to represent McFarland standard of 3, using a total volume of 30ml (Fig. 2.1). Thereafter, multiple 1ml aliquots of dilutions representing 10^6 and 10^7 cells were centrifuged and cell pellets frozen until extractions were done. At the same time, and enabling cell enumeration, aliquots from each serial dilution were spread-plated on agar and cultured anaerobically for 2 days on blood agar plates (2% horse blood, Brucella base). Each dilution was plated in triplicate. Colonies were counted on three consecutive dilutions, and the mean number of cells (and standard deviation) obtained were calculated using the Descriptive Statistics function in Excel (Microsoft365, Excel version 2402).

Initially, and once off only, 1ml aliquots of each dilution were centrifuged and the supernatant fraction (except 200 µl) was removed. Attempts were made to isolate DNA from every dilution, although no pellets were visible for dilutions below 10^{-2} of the original McFarland 3 suspension. As expected, no DNA was detected with UV spectrometry (at 260 nm). As there

was no evidence that extractions failed (as we saw no pellets), or potentially the number of cells were too low and thus yielding undetectable amounts of DNA, we abandoned extractions from any dilution higher than 10^{-2}. Going forward, DNA was extracted from neat (McFarland 3) and 10^{-1} dilutions (evidence of pellets, thus if no DNA was detected, clearly extractions failed).

To evaluate locally available commercial kits, DNA extractions were performed, in triplicate using three commercial extraction kits. These kits are all available on the local market, yet not tested with the specific pathogens used in this study (at least, at the time of thesis submission, no public information is available online).

The three kits vary in their mode of DNA purification as noted in Fig. 2.2, representing solid phase purification via spin columns, magnetic bead binding and purification of DNA and, lastly, chemical precipitation via salting out. Kit A (Quick DNA Faecal/Soil Microbe Miniprep kit (Cat nr D6010, Zymo Research, USA) employs proprietary Zymo-Spin™ Technology), claims to filter and remove humic acids and other polyphenols from DNA containing solutions. Kit B (Mag-bind® Environmental DNA 96 kit (Omega Biotek, USA)) employs the magnetic bead technology, where DNA attaches reversibly to magnetic beads coated in a proprietary substance that binds to nucleic acids (205). Kit C (Wizard® Genomic DNA purification system (Promega, USA) uses a chemical clean up and alcohol precipitation method.

The protocols for these kits are available online at:

- Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (zymoresearch.com) (Kit 1)
- https://ensur.omegabio.com/ensur/contentAction.aspx?key=Production.4023.S2R4E1A3.20190920.67.4679917 (Kit 2)
- Wizard(R) Genomic DNA Purification Kit Technical Manual #TM050 (promega.com) (Kit 3)

The protocol for each kit was followed, except that DNA was finally diluted in 40 µl and not in the elution volume prescribed by the kits. Furthermore, kits were also compared for ease of use, equipment required for extraction, number of steps and cost of kits. The presence/absence of steps to remove PCR inhibitors were also noted. Finally, DNA extracted using Kit 1 were also used to do isothermal amplification and lateral flow detection assays. Kit 1 was also used to do some preliminary RPA-LFA testing of DNA extracted from a limited number of different water matrices (as indicated in Table 2.1).

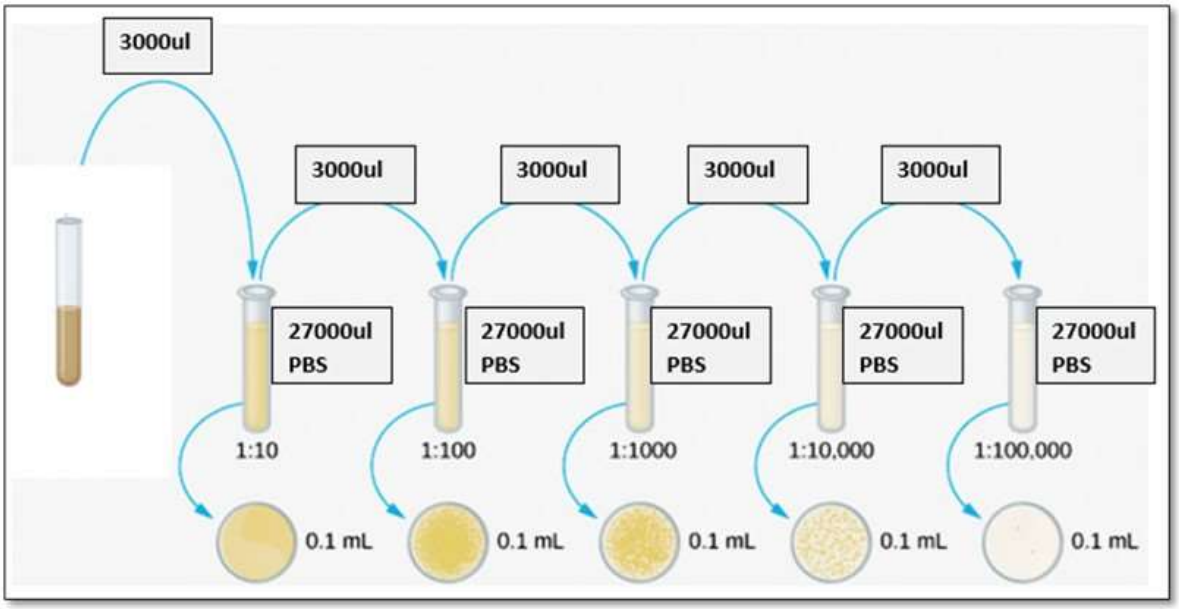

Fig. 2.1. Workflow to prepare serial dilutions of bacterial cells for enumeration and DNA extractions.

C. perfringens cells used were one day old, thus the majority of cells were in vegetative form. Cell pellets from all dilutions were stored (1ml aliquots centrifuged and pellets frozen). However, DNA was extracted only from the first two dilutions, but all dilutions were to determine the cell numbers represented by a suspension of McFarland 3). PBS, phosphate buffered saline

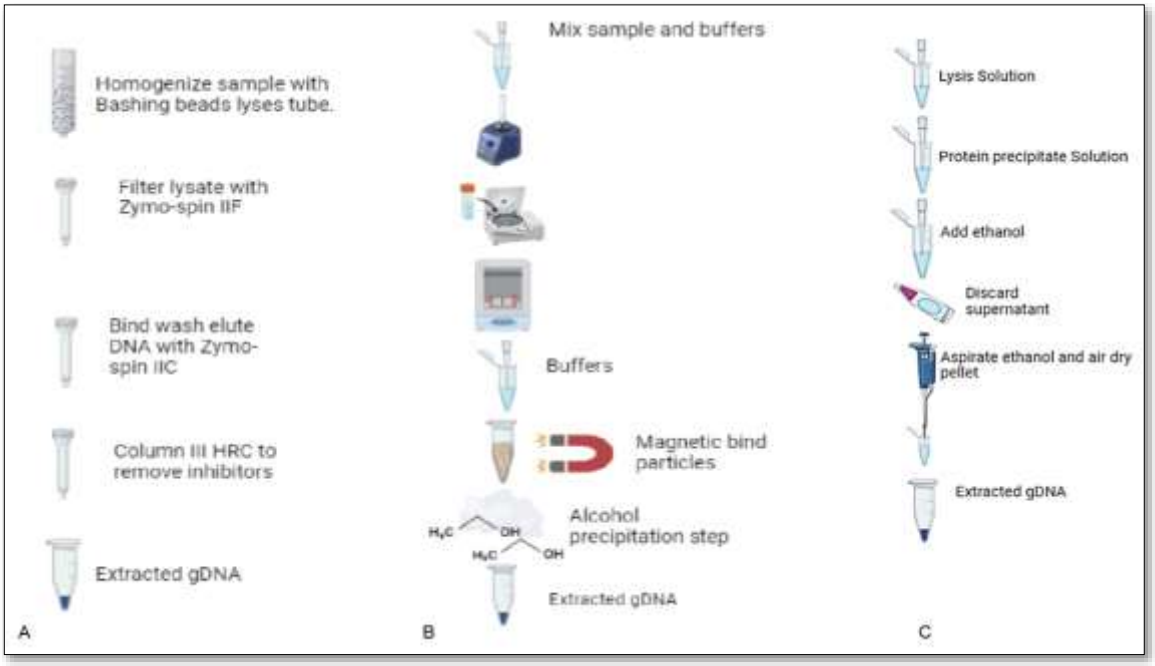

Fig. 2.2. Workflows for the three DNA extraction kits.

Kit A is the Quick-DNA Fungal/Bacteria Miniprep kit, kit B the Mag-bind® Environmental DNA 96 kit and kit C the Wizard® Genomic DNA Purification kit. An additional column is used finally in Kit A, to remove PCR inhibitory substances (column not shown). Detailed kit workflows and components are found in the links shared in Section 2.2.2.

TABLE 2.1. COMMERCIAL KITS USED IN THE STUDY

QuickDNA Fecal/Soil Microbe Miniprep Kit (Zymo Research)	Mag-Bind® Environmental DNA 96 Kit (Omega Biotek)	Wizard Genomic DNA Kit (Promega)
Spin columns	Magnetic beads	Chemical clean-up:
Mechanical disruption and lysis reagent	Mechanical disruption and lysis reagent	Chemical lysis, excluding mechanical disruption Vortex
Via Zymo-Spin™ III-HRC Filter; proprietary information, but claims to remove inhibitors	Proprietary cHTR reagent -remove humic acid and unspecified other PCR inhibitors.	Not listed in reagents
Max 80 min	90 to 120 min	45 to 60 min

2.2.3 DNA extraction from municipal sources

The Quick-DNA Fungal/Bacteria Miniprep kit (Kit A) was used to extract DNA from the water samples. Samples provided were from a municipal swimming pool, a local tidal pool, a standpipe (used for water tankers), a river, post-treatment water for potential reuse, treated wastewater and an industrial wastewater sample. The swimming pool sample contained sodium thiosulfate to remove residual chlorine prior to extractions. It was provided with the compound already added, with a standard concentration of 0.2% W/V added. For extractions, samples were filtered via 0.45 µm nitrocellulose filters (50mm diameter, Millipore), a sterile, reusable Nalgene filtration device, and a vacuum pump. Filters were placed inside 15 ml polypropylene tubes, and the filtrates were washed from membranes using PBS and vortexing. One millilitre of the vortexed fluid was centrifuged (5 minutes, 6 000 rpm, Labnet Prism Mini Centrifuge), and the pellets were re-suspended in 200ul PBS, followed by DNA extractions using Kit A. DNA was eluted in 40 µl of the kit elution buffer. An aliquot of the DNA (10µl) was removed and set aside; the rest was purified from any PCR inhibitors using the special HRC column provided in the kit. For this, the column is first washed with a provided buffer, thereafter, the DNA eluate is added to the column, centrifuged for a minute and the inhibitor-free DNA is collected in a microcentrifuge tube.

The DNA from these municipal samples were then subjected to amplification and the amplicons were detected using agarose gel electrophoreses (for 16s rRNA amplicons) and lateral flow (for RPA generated amplicons).

2.3. DNA amplification and target validation using conventional PCR

2.3.1 Gene targets and primers used in study

Genes unique to *C. perfringens* and *L. pneumophila* was amplified using conventional PCR or RPA. All primers used in this study are listed in Table 2.2. For conventional 16s rRNA PCR to confirm identity of strains used in this study, previously designed primers were used. For recombinase polymerase amplification, a new primer set was designed the *C. perfringens* plc gene, while previously described primers were employed to detect the *L. pneumophila mipA* gene. Primer design for *plc* is described in Section 2.4.

2.3.2 DNA target confirmation using conventional PCR

Conventional PCRs were done using a standard PCR reaction mix and the Promega GoTaq PCR reagents (Promega, USA) (Table 2.3). The PCR cycling conditions are described in Table 2.4. For DNA identification, the universal 16s rRNA gene primer set (Table 2.3) was used to generate amplicons that were subjected to Sanger sequencing (Inqaba Biotec, Pretoria). PCR mixes and cycling conditions as described in Table 2.3 and 2.4, with annealing temperature of 56 °C

TABLE 2.2. PRIMERS USED FOR ANALYSIS.

Gene targeted	Micro-organism	Primers (5'-3')	Amplicon size
16s rRNA	All bacteria	27F (Forward) 5' – AGA GTT TGA TCC TGG CTC AG – 3' 1541R (Reverse) 5' – AAG GAG GTG ATC CAG CCG CA – 3'	1500
plc	*C. perfringens* *	Forward: TTAGCTTATTCTATACCTGACA CAGGGGAATC Reverse: GCAGTAACATTAGCAGGATGAT ATGGAGTA	170
mip	*L.. pneumophila* serogroup1	Forward: GACAAGGATAAGTTGTCTTATA GCATTGGTGC Reverse: CTTGTTAAGAACGTCTTTCATTT GCTGTTCGG	165
plc	*C. perfringens* *	Forward: /5DigN/TT AGC TTA TTC TAT ACC TGA CAC AGG GGA ATC * Reverse: /5Biosg/GC AGT AAC ATT AGC AGG ATG ATA TGG AGT A *	170
mip	*L. pneumophila* serogroup1	Forward: /5DigN/GA CAA GGA TAA GTT GTC TTA TAG CAT TGG TGC Reverse: /5Biosg/CT TGT TAA GAA CGT CTT TCA TTT GCT GTT CGG	165

Information obtained from (206,158), *this study.

TABLE 2.3. GENERIC PCR REACTION MIXTURE TO AMPLIFY DNA TARGETS

PCR component	1 x reaction (µl)	Final conc reagents (µM)
Promega GoTaq G2 Hotstart 2xMaster Mix	12.5	1 x concentrated
Nuclease-free water	8.5	-
Primers (Forward) (10uM)	1.5	0.6
Primers (Reverse) (10uM)	1.5	0.6
Template DNA (concentration)	1	0.95
Total	**25 µl**	

TABLE 2.4. CONDITIONS FOR SPECIES SPECIFIC, CONVENTIONAL PCR

Step	*C. perfringens*		*L. pneumophila*	
Initial denaturation	95 °C	10 min	95 °C	10 min
Denaturation step	95 °C	30 sec	95 °C	30 sec
Annealing	64 °C	30 sec	56 °C	30 sec
Final elongation	72 °C	10 min	72 °C	10 min

2.3.3 Analysis of PCR amplicons using agarose gel electrophoresis and ultraviolet light

Generated amplicons were analysed using 2 % agarose gels (w/v), prepared in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) (207). Gels contained ethidium bromide (final concentration of 0.5 µg/ml, prepared from 10 mg/ml stock (Bio-Rad, USA). Electrophoreses were done in gels submerged in 1 x TAE buffer. A volume of 3 µl of 100bp DNA ladder were also loaded to one well (specific rulers indicated in results), alongside the amplicons, to determine whether the amplicons were of expected size.

The PCR reagents (Promega) used for conventional PCR contained a dye for electrophoreses. Therefore amplicons were loaded without further addition of any dues. For amplicons generated via RPA, an aliquot of 5x DNA Loading dye buffer Blue (Promega, USA) was mixed with 8 µl of extracted gDNA and loaded into wells before electrophoreses. PCR amplicons were separated by gel electrophoresis as described in above.

Electrophoreses were conducted for 60-90 minutes, using a constant voltage of 110V, then visualised using the Gel Doc XR+ Gel Documentation System (Bio-Rad, USA) and associated software, Image Lab Tough Software (Version6.1 , Standard Edition, (Bio-Rad Inc, USA).

2.3.4 Sanger sequencing to confirm amplicon identity

Following confirmation of DNA bands of the correct size, 16s rRNA amplicons (approximately 1500 bp in size), as well as *C. perfringens* (expected amplicon size of 170 bp) and *L. pneumophila* (expected size of 165 bp) amplicons were subjected to Sanger sequencing. PCR clean-up and sequencing were done by the service provider (Inqaba Biotech, Pretoria South Africa). Chromatograms were analysed in Chromas 2.6.6 (http://www.technelysium.com.au/chromas.html) before identification using BLAST (Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Identity of at least 99% was used as the cut-off to confirm genus and species (208).

2.4 RPA development for *plc*

2.4.1 Primer design to detect *C. perfringens plc* gene.

Primers used for the isothermal amplification and detection of the *C. perfringens* phospholipase C gene (*plc*) were designed using Primer 3 (https://primer3.ut.ee/). This gene was selected as it is a gene unique to this *Clostridium* species (209,210). Primer quality parameters evaluated *in silico*: using various online software. The ability to solely bind to *C. perfringens* DNA, by using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov) software that enables a search of genetic sequences of prokaryotic and eukaryotic origin. For *plc*, secondary structure formation, primer dimer formation was checked using free online software (https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/ and Primer3Plus - Pick Primers). Specificity was checked via BLAST, to confirm only *C. perfringens* is detected.

For *Legionella pneumophila*, a previously designed RPA primer set was used (164), but BLAST was done nevertheless to confirm its specificity for this species. This set had been extensively tested for specificity previously and thus we did not check specificity.

Primers were synthesised and provided by Inqaba Biotec (Pretoria, South Africa) and reconstituted as 100 µM stocks in 1xTris-EDTA Buffer (10 mM Tris, 1 mM EDTA, pH 8.0). For PCR, working stocks of 10µM were prepared using molecular grade water. Primers were kept at -20 °C and defrosted only when PCR mixes were to be prepared.

Prior to RPA, conventional PCR were done (set up as described in Tables 2.3 and 2.4) to confirm that the correct products were amplified. Amplicons were run on agarose gels to verify the size and then sent for Sanger sequencing as described in Section 2.2. Following confirmation, RPA assays were developed.

2.4.2 RPA optimisation for *plc* gene

Recombinase polymerase amplification reactions were done using the commercial assays TwistAmp Basic (TABAS03KIT) and TwistAmp Liquid Basic kits (TALQBAS01). Reactions were set up as per the manufacturer's instruction (TwistDx™ Limited, UK). To determine the conditions for effective PCR, the following parameters were optimised: primer concentration, annealing temperature, and time.

The TwistAmp Liquid Basic kit and protocol were used for initial optimisations. Prior to setting up reactions, the heated device to be used needs to be prepared. In this study, we used a PCR instrument, set to be at 37 °C for pre-set time (maximum 40 minutes), a single cycle (unlike a typical PCR that requires multiple cycles of amplification). Then RPA mixes were

prepared and typically contained reaction buffer, dNTPs, Basic E-mix, Magnesium acetate (MgOAc), primers and DNA template. Briefly, a master mix was prepared in one tube as follows: reaction Buffer (25 µl), dNTPS (9.2 µl of 10mM stock) plus 5 µl of 10 x Basic E-mix. This was mixed via a pulse vortex, and centrifuged briefly to collect liquid at the bottom of PCR tubes. Thereafter, 2.5 µl of the 20x Core Reaction Mix were added (to the tube lid), the tubes closed, inverted, and spun briefly again in the micro-centrifuge. The primers (2.4 µl each of 10 µM stocks) are added to 41.7 µl of this master mix, thereafter the DNA template (1µl volume) and 2.5 µl of MgOAc (280 mM, supplied in kit) were added to tube lid (this reagent is essential co-factor for the polymerase action and thus added to lid to prevent amplification starting before the tubes are placed in heated device). The entire mixture was inverted maximum 15 times, then immediately transferred to the heated device as amplification starts as soon as all reagents are mixed together. Note that this mixture is fairly viscous prior to amplification. The amplification protocol was as follows: Amplification was done at a temperature of 37 °C for 20 minutes using the thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, USA) as the heat source. According to the manufacturer, 37°C is the minimal temperature to use for amplification

To optimise the RPA, the **lowest amount of DNA** to amplify was first evaluated by adding decreasing amounts of DNA template to reaction mixtures (10, 20, 40 and 50ng per reaction, each in a total of 1 µl).

Hereafter, **optimal time** to generate amplicons were evaluated at 15, 20, 30 and 40 minutes, each at 37 °C. For this, a standard amount of 20 ng of *C. perfringens* DNA was added per reaction. Reactions were mixed at the same time but kept on ice until it was put into the PCR machine.

Finally, the **optimal temperature** was determined by testing the assay at 37 °C, 38 °C, 39 °C, and 40 °C for 30 minutes. Again, a standard amount of 20 ng of DNA was used per reaction.

The abovementioned setup was done in initial RPA development, using the Liquid Basic kit. However, the extreme viscosity of one of the reagents posed technical difficulty for reliable pipetting, plus the fact that reagents are supplied in “bulk” (each buffer in one bottle meaning having to freeze-defrost repeatedly was deemed problematic. Therefore, further work was done using the TwistAmp Basic kit[2], consisting of rehydrated reaction mixes of reagents,

[2] https://www.twistdx.co.uk/wp-content/uploads/2021/04/INTABAS-v3.0-TwistAmp-Basic-Kit-Quick-Guide-INTABAS-1-1.pdf

with only template and Magnesium acetate to be added after rehydration of the lyophilised material. The time and temperature titration experiments were repeated using the TwistAmp Basic kit.

This kit consists of lyophilised ingredients, pre-packed in individual tubes and supplied as 8 tube strips. An investigator just needs to rehydrate the lyophilised ingredients, which is all the RPA reagents except the primers, DNA template and MgOAc. Rehydration is done using a supplied buffer, set up as indicated in Table 2.5. Thus, setup is less cumbersome and less prone to technical variation, as we have found with the 20x Core reaction mix of the Liquid Basic kit.

TABLE 2.5. REACTION MIXTURES FOR RPA REACTIONS USING THE BASIC KIT

Reagent (concentration)	Volume per reaction
Primers (10 µm)	2.4 µl
Primer free rehydration buffer (provided in kit)	29.5 µl
Template	13.2 µl

2.4.3 Limit of detection using conventional PCR and RPA

For limit of detection experiments, stock solutions (10 ng/µl) of DNA extracted from *C. perfringens* and *L. pneumophila* were prepared in PBS. Using these stock solutions, tenfold serial dilutions of the DNA ranging from 1 ng.µl^{-1} to 1x10^{-6} ng.µl^{-1} were prepared in a total volume of 50 µl by mixing 5 µl of gDNA with 45 µl of PBS buffer.

2.4.4 Amplicon detection via UV fluorescence on agarose gels

For visualisation of RPA amplicons on agarose gels, an aliquot of amplicons was mixed with a 5x DNA Loading dye buffer Blue (Promega, USA) and run on agarose gels as described in Section 2.3.3.

2.4.5 Lateral flow detection of dual labelled amplicons

To detect amplicons using a lateral flow device, labelled primers (Table 2.2) were used to generate dual labelled amplicons. To enable detection, for both *mipA* and *plc*, the reverse primers were labelled with biotin, while the forward primers were labelled with digoxigenin (DIG). These dual labelled amplicons generated from pure DNA of *C. perfringens* and *L. pneumophila* was then captured and visualised via a commercially available lateral flow device (PCRD device, Abingdon Health UK). The mechanism of detection is described here: IFU-PCRD-Nucleic-Acid-Lateral-Flow-Immunoassay-v5.0.pdf (abingdonhealth.com) and is a typical sandwich immunochromatographic assay (also see Fig. 2.1). Please note that detection of the two bacterial targets was done on individual strips. Following RPA amplification, if dual labelled amplicons were generated, the DIG-labelled end of amplicons are captured by the anti-DIG antibody that is embedded on the PCRD device on the relevant strip. Captured amplicons become visible on the test line (T) as shown in Fig. 2.1)

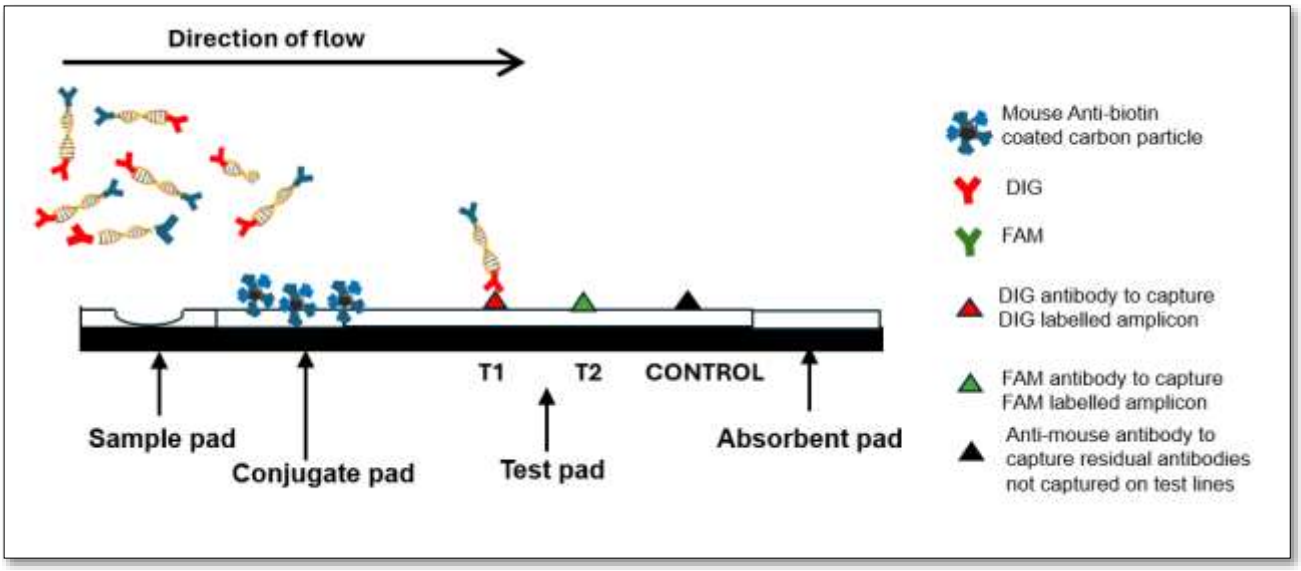

Fig. 2.1. Mechanism of detection of dual labelled amplicons captured on a commercial lateral flow strip.

The PCRD device as illustrated above, contains an antibody against DIG and 5-FAM, enabling capture of DIG/biotin and FAM/Biotin dual labelled amplicons at test lines 1 and 2 respectively. Dual labelled amplicons are mixed with the supplied LFD buffer, applied to the sample pad, from where it flows, via capillary action towards the conjugate pad, test pad and absorbent pad. At the conjugate pad, it rehydrates the Neutravidin coated carbon particles ( ) this interacts with the Biotin label on the 3' end of the amplicons and then migrates towards the test pad. Here it will bind to test line 1 (will capture amplicons with a DIG label) or test line 2 (capture amplicons with a 5-FAM or FITC label). The concentration of the captured amplicons in one space leads to the appearance of a line visible to the naked eye. In our assays, both amplicons are labelled with DIG.

2.5 Testing the RPA-LFD detection workflow using real world samples.

Prior to doing the RPA, conventional 16s rRNA PCR was done to discern if there was bacterial DNA present in the samples. PCRs were set up with 16srRNA primers as described in Table 2.2 and cycling reactions as described previously. DNA extracted from water samples was then tested for the presence of *C. perfringens* and *L. pneumophila* DNA using RPA. RPA reactions were set up as described in Table 2.5, using the respective *mipA* or *plc* labelled primers. Following amplification for 30 minutes, an aliquot of the RPA reaction mixture (6 µl) was mixed with the provided PCRD buffer (84 µl), mixed and 75 µl loaded on the PCRD device. Development of colour at the test line for DIG was monitored over a period of ten minutes (As per instructions, results must be read within 10 minutes).

2.6 Ethical considerations

This project did not require animal or human ethics as no human or animal material was used, and neither was human subjects involved in the study. UCT Faculty Biosafety Approval was acquired FBC REF.: LP/004a/2021-2024 and Ethics Declaration in Appendix B.

CHAPTER 3: RESULTS

3.1 Confirmation of identity of reference material.

Generated 16s rRNA amplicons were subjected to Sanger sequencing. Analysis of generated nucleotide sequences via the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed identity of amplicons as originating from *C. perfringens* and *L. pneumophila* respectively (more than 98% homologous identity). Therefore, the cultured material was correct (chromatograms and BLAST results not shown, available if requested)

3.2 Primer design and confirmation of *C. perfringens plc* primers.

Targeting the *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/). The primers were tested for the ability to bind only *C. perfringens* DNA, by BLAST analysis (https://blast.ncbi.nlm.nih.gov), checking the sequence against all available microbial genomes in this free online database. Following this, the most ideal pair predicted by Primer3 were ordered from Inqaba Biotec (Pretoria, South Africa). Primers were tested first with conventional PCR to verify that it amplifies the correct size product. The size of the generated amplicons (170 bp) was correct (Fig. 3.1). Different amounts of DNA template were used (lanes 2-6), with the lowest amount of (5 ng, lane 2) successfully generating a single amplicon as well, with no non-specific amplification. Furthermore, Sanger sequencing verified that the correct gene target (*plc*) was amplified.

3.3 Verification of previously designed primers for *L. pneumophila mipA*

Previously described RPA primers and PCR conditions were used (158). Primers were tested first with conventional PCR to verify that it amplified the correct size product. The conventional PCR generated the amplicon of the correct size (165 bp) (Fig. 3.2) and identity confirmed with Sanger sequencing as well.

Having shown that the previously published *mipA* primers, as well as the newly designed new plc primers indeed generated the correct amplicons (and verified further with Sanger sequence analysis), we proceeded with further work. Before further isothermal PCR development was done with the abovementioned primers, we first evaluated different DNA extraction kits to discern which would generate DNA from the pure, cultured cells of C. perfringens. We opted to test the kits with this microbe as its cell wall is known to make DNA extractions trickier compared to other microbes

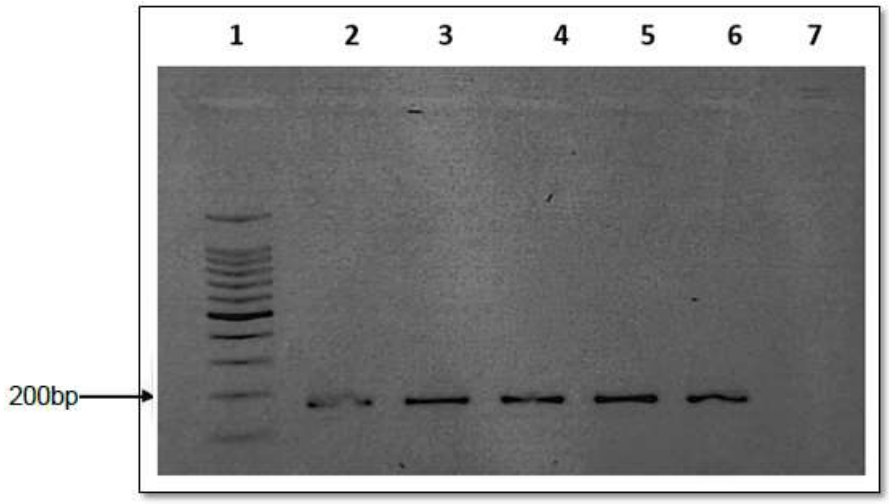

Fig. 3.1. Agarose gel confirmation of *C. perfringens plc* internal fragment amplification and size.

Agarose gel electrophoresis shows that the newly designed primer pair successfully generated amplicons of the correct size (170 bp), with no non-specific amplification. Lane 1, DNA size marker (Generuler 100 bp ladder, Thermo Scientific), Lanes 2-5, C. perfringens amplicons generated with input DNA of 5,10, 20,40 and 55 ng of DNA, Lane 7, negative control (water). Amplicons were generated using GoTaq Mastermix, and the primers hybridization temperature were 64°C

3.4 Comparison of extraction kits using pure, cultured strains

C. perfringens is a Gram positive organism with a cell envelope that is known to impact DNA extraction efficiency. Furthermore, it also produces spores that are hardy and problematic for DNA extractions. Therefore, we evaluated the quality and quantity of gDNA extracted from *C. perfringens,* using three commercially available DNA extraction kits (Table 3.1). Younger cultures (log phase cells) will likely contain mostly vegetative cells and some spores, whereas older cultures will predominate with spores. One day old, cultured cells (on Blood agar) were standardised using are McFarland standard of 3, and colony counts showed this density contained an average of approximately 8.25x106 cfu/ml (rounded off to $1x10^7$ cells). As expected, Gram stains of cultures showed mostly vegetative cells and some spores (data not shown).

The rationale for the three selected kits were due to the fact that the kits use three different modes of DNA purification. Furthermore, two of the kits also contained an additional step to remove potential PCR inhibitors from samples (useful for testing water with potential PCR inhibitors). Secondly, none of these kits are currently reported in the local literature for use in water pathogen research, although two of the kits claim to be targeted at environmental pathogens.

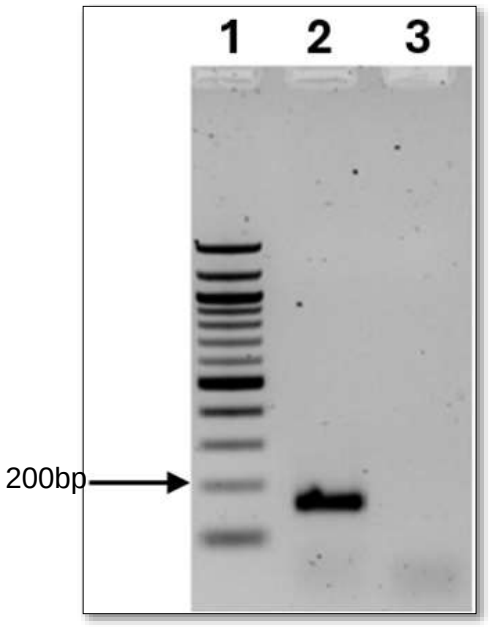

Fig 3.2. Conventional PCR targeting the *L. pneumophila* a *mipA* gene.

Lane 1, MWM (NEB Quickload 100bp ladder); lane 2, *mip* PCR with *L. pneumophila* template; lane 3, negative control, with water as a template.

Yield and purity of DNA. DNA was extracted from a standard number of bacterial cells (approximately 8.6 x 10^6 cfu/ml) and eluted in 40 µl of elution buffer. Comparing the yield of DNA, the Quick-DNA Fungal/Bacteria Miniprep kit was most consistent, while the yield from the other two kits fluctuated more as indicated in Table 3.1.

Regarding the purity of DNA, as measured by the 260/280 ratio, both the Quick-DNA Fungal/Bacteria Miniprep kit and Wizard® Genomic DNA Purification kit values were below the index of 1.8-2 (indication of pure DNA) (Table 3.1). A value below 1.8 generally indicates residual contamination from proteins or chemicals such as phenol and other kit reagents (211).

TABLE 3.1. YIELD AND PURITY OF EXTRACTED GENOMIC DNA FROM *C. PERFRINGENS*

Sample	Yield of DNA (ng/µl)	Total yield of DNA eluted in 40/µl (ng)	260/280	260/230	Kit used
Replicate 1	17.8	712	1.55	0.06	Quick-DNA Fungal/Bacteria Miniprep kit (A)
Replicate 2	14.4	576	1.76	0.44	
Replicate 3	16.6	656	1.23	0.03	
Average	**16.27**	**648**			
STD deviation	**1.41**	**55.80**			
Replicate 1	41.1	1644	1.64	0.06	Mag-bind® Environmental DNA 96 kit (B)
Replicate 2	14.4	576	2.19	0.04	
Replicate 3	13.8	552	2.38	0.03	
Average	**23.1**	**564***			
STD deviation	**12.73**	**12.00**			
Replicate 1	41.8	1672	1.5	0.37	Wizard® Genomic DNA Purification kit (C)
Replicate 2	63.3	2532	1.44	0.46	
Replicate 3	78.8	3152	1.47	0.46	
Average	**61.30**	**2452**			
STD deviation	**15.17**	**606.84**			

*average calculated from 2 replicates; other 2 kits average yield calculated from 3 replicates

Overall, the Wizard® Genomic DNA Purification kit yielded on average (41 – 78 ng/µl), though more variable compared to the spin-column purification method (more reproducible, less technical variation but lowest yield) and the magnetic bead purification method (highest yield, but one sample an outlier and thus more technical variation. If the outlier is excluded from mean calculations, then the spin column kit yielded overall higher concentration of DNA, though not much different from the magnetic bead kit.

In terms of purity the absorbance at 260/280 nm for all three kits gave values below 1.8 with exception of two extracts which obtained ratios of 2.19 and 2.39 when the Mag-bind® Environmental DNA 96 kit was used.

Workflow and ease of use of kits. As indicated in Table 3.2 below, the kits were compared in terms of ease of use, equipment needed, heat treatment, time to completion of procedures, the need for mechanical lysis and clean-up method. Kit B required the most amount (39 steps, 90-120 minutes) compared to Kit C (19 steps, 45-60 minutes) and Kit A (10 steps, 80 minutes).

All three kits required an electrical source to operate a micro-centrifuge that could handle micro-centrifuge tubes, while Kit B and C required heat treatment (we used a heating block); in this regard Kit A did not require heat treatment. Only Kit C did not utilize mechanical to lyse *C. perfringens.* This kit also does not require columns or magnetic bead devices, which is additional steps for clean-up. However, as the DNA is precipitated via alcohol precipitation in this kit, it takes longer to finish the extraction process as DNA extracts needs to be precipitated, air-dried, and then rehydrated. This is a negative trait, compared to the other two kits that generated ready-to-use.

We conclude that Kit A was the easiest, fastest and required the least technical experience. While Kit B was also easy to use, the excessive number of steps (notably repeated vortexing steps) is not conducive as more steps pose an increased risk for technical errors and is more time consuming. Also, although Kit C is a simple procedure, but the need to precipitate and rehydrate the DNA is not good for rapid POC testing.

3.5 Development of recombinase polymerase amplification for *plc* and *mipA* genes

A commercial RPA kit (RPA Basic, TwistDX) kit was used to test a previously described RPA assay for *L. pneumophila mipA* gene and *plc* gene from *C. perfringens.* We first used the Liquid Basic kit, then changed to the lyophilised Basic kit (which consists of lyophilised premixes). This was decided for the following reasons:

The Liquid Basic kit supplies reagents in bulk. All reagents need to be stored frozen and individual ingredients defrosted and mixed for PCR setup; this is cumbersome. The individual ingredients thus had to be exposed to repeated cycles of frosting-defrosting. This is not good practice, unless bulk reactions are set up (the number of defrosting cycles thus reduced and reagents used up sooner).

One of the supplied buffers contains an ingredient (a crowding reagent) making it extremely viscous; this proved to be problematic for reliable pipetting and results. This reagent assist with efficient amplification, and thus, is necessary.

To circumvent the repeated cycles of frosting-defrosting, all ingredients, except the buffer containing the crowding reagent, were aliquoted into smaller volumes sufficient to set up batches of 5 reactions. These were frozen and only defrosted when needed. This resulted in prolonging of the reagent life span.

TABLE 3.2. COMPARISON OF GENOMIC DNA EXTRACTION KITS.

VARIABLE	KIT A	KIT B	KIT C
Pre-treatment, cell lysis and homogenization	Mechanical lysis (Bashing Beads ™) Vortex, bead beating Lysis Buffer (contain Guanidinium thiocyanate, hazardous) Vortex	Mechanical lysis (Ceramic Beads) Vortex (multiple steps) Lysis Buffer (contain Guanidinium thiocyanate, hazardous)	No beads, no mechanical lysis Chemical Lysis alone Lysis buffer ingredient not specified, but non-hazardous according to MSDS sheet
Endolytic enzymes	None used according to kit	Lysozyme	Lysozyme
Clean up method	Silica based Spin column used to 1.Bind nucleic acid and separating it from cell debris and chemicals 2. Nucleic acid cleaned via washing with buffers 3. Purified DNA eluted from column in EDTA containing solution	Clean-up using repeated wash steps: Magnetic beads bind nucleic acids Liquids removed via pipetting Repeated washing steps, binding to beads Finally, pure DNA eluted from beads	Chemical clean-up: Proteins removed via precipitation and centrifugation DNA recovered by alcohol precipitation and reconstitution in TE
Adsorption of inhibitors	Proprietary HRC columns, name of matrix not listed in reagents	Proprietary cHTR reagent - remove humic acid and unspecified other PCR inhibitors.	Not listed in reagents
Approximate time to completion	Max 80 min	90 to 120 min	45 to 60 min
Number of steps	10	39	19
Average cost of kit [3]	Below 10K ZAR	Above 10K ZAR	Below 10K ZAR
Additional reagents not supplied by kit	Ethanol	Ethanol	Ethanol
Heat treatment	None	Yes, with heating block	Yes, with heating block
Equipment required	Bead beater Centrifuge	Centrifuge Magnetic separation device Incubator Ice bucket	Centrifuge Incubator

However, this is not fit for work done away from a laboratory, the extreme viscosity of the one reagent will be problematic for less experienced technicians and we observed technical difficulties in beginning with accurate pipetting; thus, we did not proceed with this kit. Instead, we opted to use the Basic kit (containing lyophilised ingredients conveniently prepared in strips of 8 tubes). Each tube contains all RPA reagents and just needs to be rehydrated with the provided Rehydration buffer, primers, template and the MgOAc. Thus, set-up is more speedily, the pipetting error associated with the dense crowding agent-containing buffer avoided. In terms of error, there are fewer steps to perform and fewer opportunities for human error.

[3] Cost of kits at time of thesis submission in 2024

So, thus we optimised PCR conditions using the liquid Basic kit, but then switched to the lyophilised Basic kit for all other work. Firstly, we evaluated if we could do the RPA successfully by using a previously published, optimised method to detect the *mipA* gene from *L. pneumophila.* Following that, and showing we could repeat the published results, we designed an RPA for another microbe that could cause waterborne disease, namely *C. perfringens.*

3.5.1 RPA for the *L. pneumophila mipA* gene

RPA for *L. pneumophila* was set up as reported previously described (158) and generated an amplicon of the correct size as previously reported (i.e., 165 bp). Genomic DNA obtained via Kit A (Quick DNA Faecal/Soil Microbe Miniprep kit) was used for RPA reactions. Non-specific smearing was noted in the control (lane 3). The amplicons shown below in Fig. 3.4 were loaded without being cleaned up, so that any non-specific effects can be visible. Identity was further confirmed via Sanger sequencing. It should be noted that RPA at 38 °C did not work well; we ascribed it to potential technical error as we found the Liquid Basic kit problematic to work with in terms of reliable pipetting of the one buffer. However, as can be seen, the previously reported *mip*A RPA was done successfully in our hands, using 30 ng of input DNA.

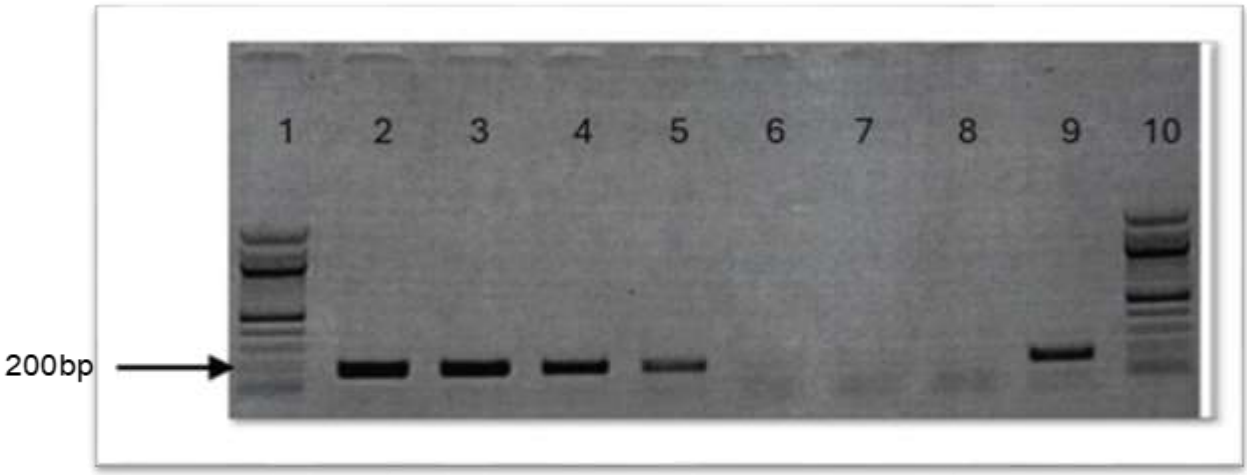

Fig. 3.3. Detection of *mipA* labelled amplicons generated via conventional PCR.

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 DNA ladder (Quickload, 100bp,NEB); Lane 2: 10 ng input DNA, Lane 3: 1 ng input DNA; Lane 4: 0.1 ng input DNA, Lane 5: 0.01 ng (10pg) input DNA; Lane 6: 1pg input DNA; Lane 7: 0.1pg 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**.**

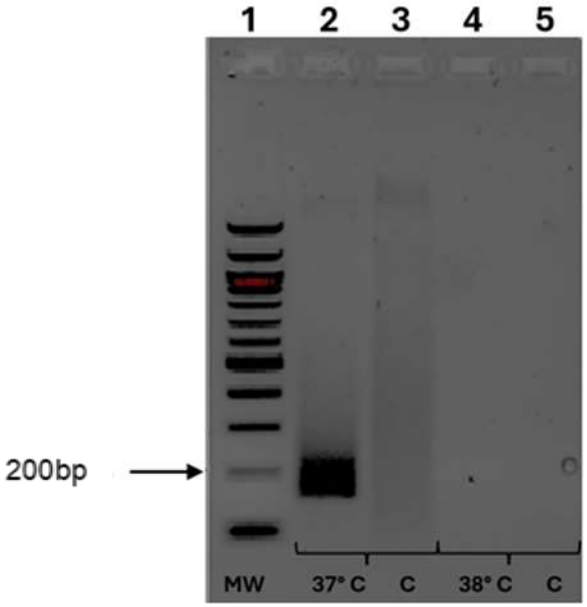

Fig 3.4. RPA generated mipA amplicons using temperature titration

Lane 1, MW marker, (Quickload 100 bp). Lanes 2-5, PCR was done at an indicated temperature below gel; lanes 2 and 4 contained input DNA of approximately 16ng/PCR; lanes 3 and 5 contained PCR with water = negative control PCRs. Other temperatures were not tested as this primer set was validated previously (157)

3.5.2 Lateral flow detection of dual labelled amplicons

Dual labelled amplicons were generated to enable capturing on a lateral flow device pre-manufactured with antibodies against DIG, 5-FAM and biotin. We opted for primers modified with biotin and digoxigenin (DIG), and again verified first that it generates amplicons of the

correct size (as evidenced by the presence of amplicons of correct size on gels). Reverse primers for both microorganisms were modified with biotin, while the forward primers were labelled with DIG (for *C. perfringens plc* and *L. pneumophila mipA* genes).

These labelled primers were also purchased from Integrated DNA Technologies (IDT, https://eu.idtdna.com/pages/products/custom-dna-rna/dna-oligos), but are available from other local suppliers as well (all using overseas manufacturers and importing the primers). According to the PCRD protocol, it does not matter which label is on which primer, as long as it generates a dual labelled product that can be captured by the antibodies present on the device (https://www.abingdonhealth.com/products/pcrd/#use).

3.5.2.1. Lateral flow detection from dual labelled amplicons generated via conventional PCR of *L. pneumophila mipA*

Amplicons were detected in both agarose gels and the lateral flow strips when conventional PCR was used to generate dual labelled amplicons (Fig. 3.3 and 3.5). For *L. pneumophila*, labelled amplicons generated with conventional PCR were clearly visible when as little as 0.01 ng of DNA (10 picogram, Fig. 3.3 lane 5) was used. On the agarose gel, a very faint band (barely visible on the gel but seen by the eye) was detected in lane 6 (input DNA 1, even at (1x 10^{-3} ng (1 pg) of input template (Lane 6). No non-specific amplification was visible. The same amplicons were loaded on the PCRD device, specifically only amplicons generated with 10 ng, 1 ng, 10 pg amplicons (in our hands 10ng input DNA consistently generated amplicons so we used that as a control). As seen in Fig 3.3 and 3.5, amplicons from as little as 10 pg of template were detected, although a very faint band was detected with amplicons from 1ng of input DNA. This was more visible on the lateral flow device. (Fig. 3.5). Further testing is needed to verify the lower limit of detection for conventional PCR (not in the scope of this study as we focused on RPA).

3.5.3 RPA and lateral flow detection of *mipA* gene from *L. pneumophila*

RPA was done at 37 °C, using serial titration of DNA, the lyophilised TwistAmp Basic kit and

Fig. 3.5. Lateral flow detection of *L. pneumophila* following conventional

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 pg (control), -3 = 10pg DNA; -4 = 0.1 pg) were loaded on strips.

labelled primers. RPA done with as little as 10 pg of template DNA generated visible amplicons (Fig. 3.6, Lane 6), input of 1 pg also generated a visible band, but with more smearing present. Lower than that, clearly defined bands (as seen in lanes 2-5) are not visible as more smearing appeared as the amount of input DNA decreased. Amplicons were not cleaned up prior to loading on the gel, hence contained unincorporated reagents and non-specific products.

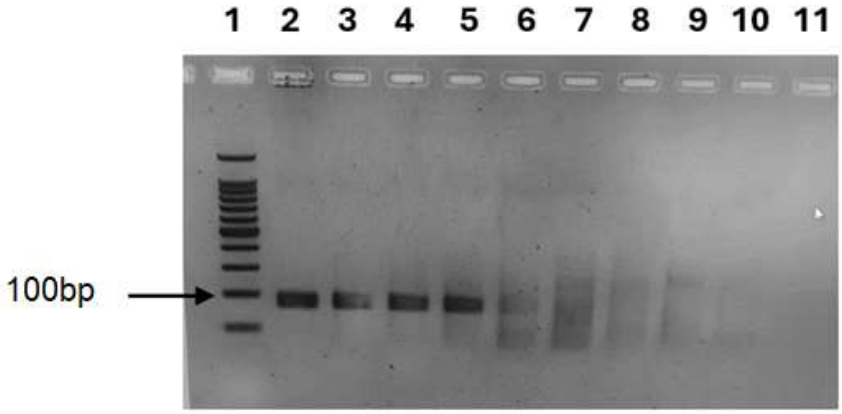

Figure 3.6. *mipA* RPA amplicons from *L. pneumophila* DNA titrations.

Lane 1, MW marker, (Generuler 100 bp). Lane 2, RPA with 30ng of input DNA (positive control); Lanes 3-9, PCR done at using 10-fold serially diluted DNA (10ng to $1x10^{-6}$); Lane 10, RPA done with water = negative control.

The same amplicons were loaded onto the LFD lateral flow device (Fig. 3.7). Clear colour development was seen right up to 1 pg of input DNA, after this, a faint band/smearing was observed for all lower dilutions and the negative control, suggesting that the non-specific amplification/smearing seen in the agarose gels also were captured at the specific antibody (thus the very faint bands on the LFD). This indicates that the lower limit of detection needs to be carefully interpreted and likely is negative (looks similar to negative control). The experiment was done twice, and representative picture was shown.

Fig 3.7. Lateral flow detection of labelled RPA mipA amplicons

30, 30 ng template DNA used in RPA= positive control, 10,10 ng input DNA, -1 to -6, = 10-fold serial dilutions of DNA, from 1 ng to 0.01 pg DNA used. C. LFD control line, should always be positive, indicating that the strips are functioning correctly. N, negative control

We demonstrated successful RPA amplification and detection of labelled amplicons using a previously described RPA primer pair for the *L. pneumophila mipA* gene, with lowest amount clearly visible on a gel when input DNA as low as 1 pg is used for amplification.

3.5.4 RPA development for the *C. perfringens plc* gene

To discern the optimal time to generate amplicons, the initial protocol followed was as per the TwistAmp Liquid Basic kit INLQBAS-v4.0-TwistAmp-Liquid-Basic-Quick-Guide-INLQB.pdf (twistdx.co.uk), then done using the lyophilised kit. The time to generate amplicons were evaluated first. Multiple reactions were set up and incubated at increasing reaction times, namely 15, 20, 30 and 40 minutes (plus control) (Fig. 3.8) was used. The generated amplicons were run on a 2 % agarose gel. It was concluded from the analysis that time as little as 15 minutes was sufficient to generate visible *plc* RPA amplicons.

Secondly, the optimal temperature to do RPA at was evaluated, also using the TwistAmp Liquid Basic kit. Multiple reactions were set up and incubated at increasing temperatures. The result showed that amplicons were generated at 37-39 ˚C, but non-specific amplification was observed at higher temperatures (above 37 ˚C) (Fig. 3.9).

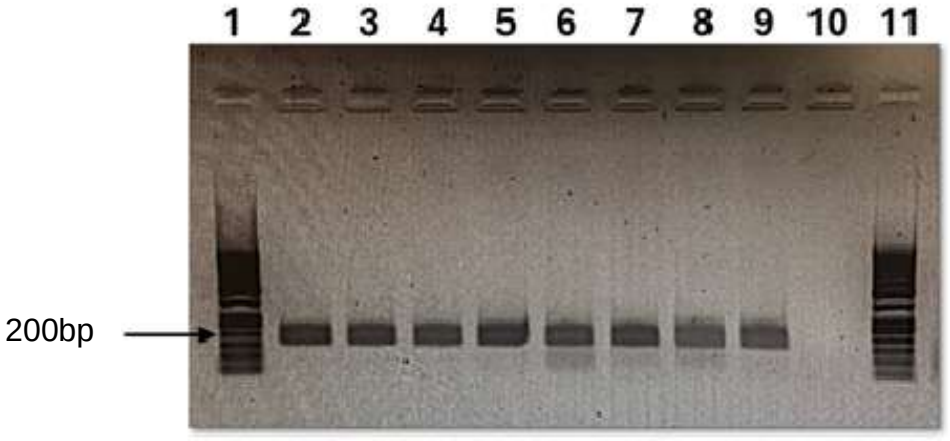

Fig 3.8. RPA amplicon generation for *plc* at decreasing time.

20ng of DNA was used per reaction. Lane 1, MW marker (GeneRuler 50 bp DNA Ladder,Thermo Scientific); lanes 2-3, 40 min amplification; lanes 4-5, 30 min amplification; lanes, 6-7, 20 min amplification; lanes 8-9, 15 min amplification; Lane 10, negative control(water instead of template). Amplification was done at 37 °C, the gel represents amplification using the TwistAmp Basic kit. The same experiment was done using the Liquid basic kit, but result was not as clear, with more smearing – ascribed to technical difficulty with pipetting of one reagent, as well as the crowding agent in kit.

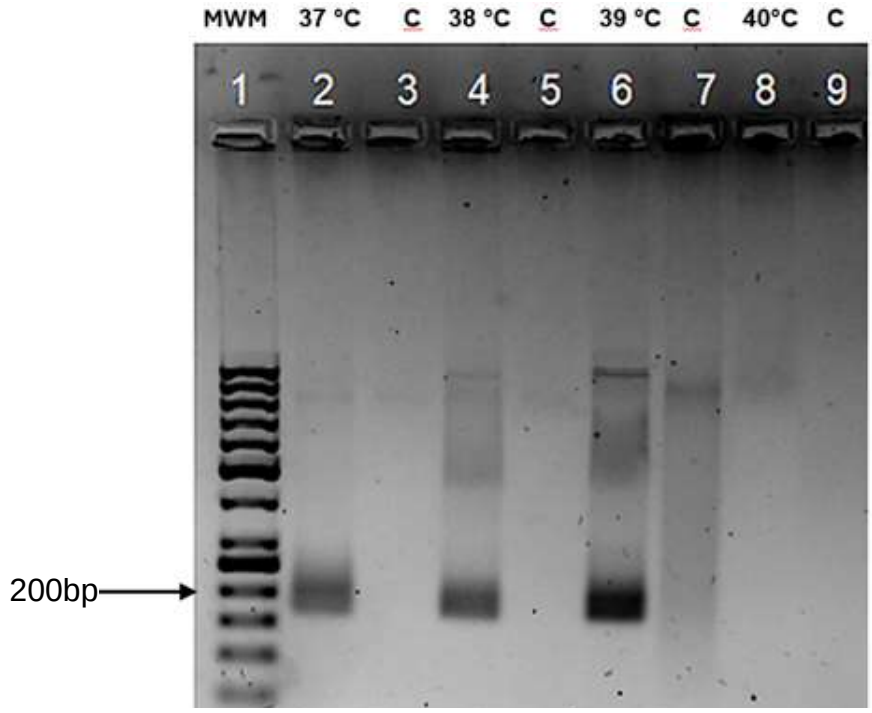

Fig. 3.9. RPA generated phospholipase C (*plc*) amplicons using temperature titration.

Lane 1, MW marker, (O' Generuler 50 bp ladder) Lanes 2-9, PCR done at indicated temperature; lanes 2,4,6,8 contained input DNA of approximately 23ng/PCR; lanes 3,5,7 and 9 contained negative control PCRs. Amplification was done using the TwistAmp Liquid Basic kit.

Note that 39 °C generated more amplicons (as visualised on agarose gel and intensity of bands) compared to other tested temperatures. However, due to technical difficulties with pipetting the highly viscous buffer in the kit in our hands, and only one replicate for each, plus the presence of non-specific smearing, no conclusion was reached regarding optimal temperature. Also, the limited visible, non-specific smearing at lower temperatures suggest that the lower temperatures might be the best temperature to do the RPA at.

3.5.5 Lateral flow detection from dual labelled amplicons generated via conventional PCR – *C. perfringens*

Dual labelled amplicons of *C. perfringens plc* (Fig. 3.10) were visible on gels with amplicons from input template as low as 1 pg DNA. As seen in Fig. 3.10, amplicons generated from as little as 1 pg of input DNA were also visible on the LFD device (similar to the gel therefore).

The non-specific smears seen in lanes 7 and 8 (Fig. 3.10), were not detected on the LFD device. We conclude that the potential lower limit of detection of the *plc* target most likely is x 1pg of input DNA, when using 37°C, the standard primer concentration

recommended by the kit, and an amplification time of 30 minutes, However this was not done with more than two replicates and a proper laboratory validation for diagnostic purposes would need to be done to determine the LOD using standard diagnostic development guidelines. Interestingly, the positive control did not amplify as well as the 10ng sample. We ascribe this to our use of a standard tube of *C. perfringens* DNA extracted on a different date and consistently used as control (was also older than DNA used in the titration this was a convenient sample that we used right at the start of the project and since it was positive we used that as the control).

Labelled amplicons generated from selected input DNA were loaded on the lateral flow device (Fig. 3.11). Amplicons generated from input DNA as low as 10 pg were detected, but not as clear as seen with the amplicons on the gel; this has to be repeated in future, using sufficient replicates to verify limit of detection. The dilutions lower than that did not give visible signals on the LFD.

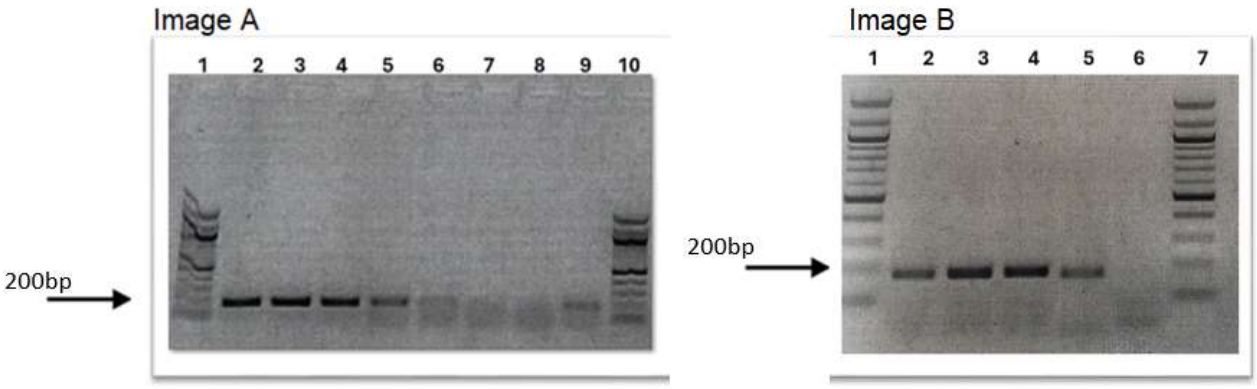

Fig. 3.10. Limit of detection of *plc* amplicons generated via conventional PCR

Serial dilutions of DNA (10 ng - 1x 10^{-7} ng of DNA) were used to prepare amplicons Gel on left (image A): Lane 1: 10 ng input DNA, Lane 2: 1 ng input DNA; Lane 3: 0.1 ng input DNA, Lane 4: 0.01 ng input DNA; Lane 5: 1 pg input DNA; Lane 6: 0.1 pg input DNA. Lane 7: 0.01 pg input DNA; Lane 8: negative control with water as template; Lane 9: positive control, 30 ng of DNA of *C. perfringens;* Lanes 1,10,and 7 Molecular weight marker, 100bp Quickload DNA ladder was used. Gel on right (image B), amplicons re-run on new gel, with amplicons from lanes 3-6 of gel on left, plus negative control).

For RPA amplicon detection on the LFD, labelled primers were used in RPA reactions. Like conventional PCR, RPA *plc* amplicons yielded bands on the LFD strips (Fig. 3.12), when lowest input DNA of 10 pg template was used. Lower concentrations 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 Fig. 3.10), thus suggest that an input of DNA lower than 10 pg cannot be detected. To verify the lower limit of detection, a proper validation, with sufficient replicates needs to be done, to

confirm the true LOD of this assay. Note again, that some faint lines appear below 10pg of template DNA amplicons, as well as in the negative control. This is suggestive that primer dimers or non-specific smearing generate this background and that more optimisation is needed. This experiment was done more than once, with the same outcome, representative picture shown.

Fig. 3.11. Confirmation of labelled *plc* PCR amplicons detection on PCRD device.

plc = gene target; 10,10ng of input DNA used in RPA, -3, 10pg DNA, -4, 1pg input DNA, -.0.01pg input DNA. Control line C should always be positive.

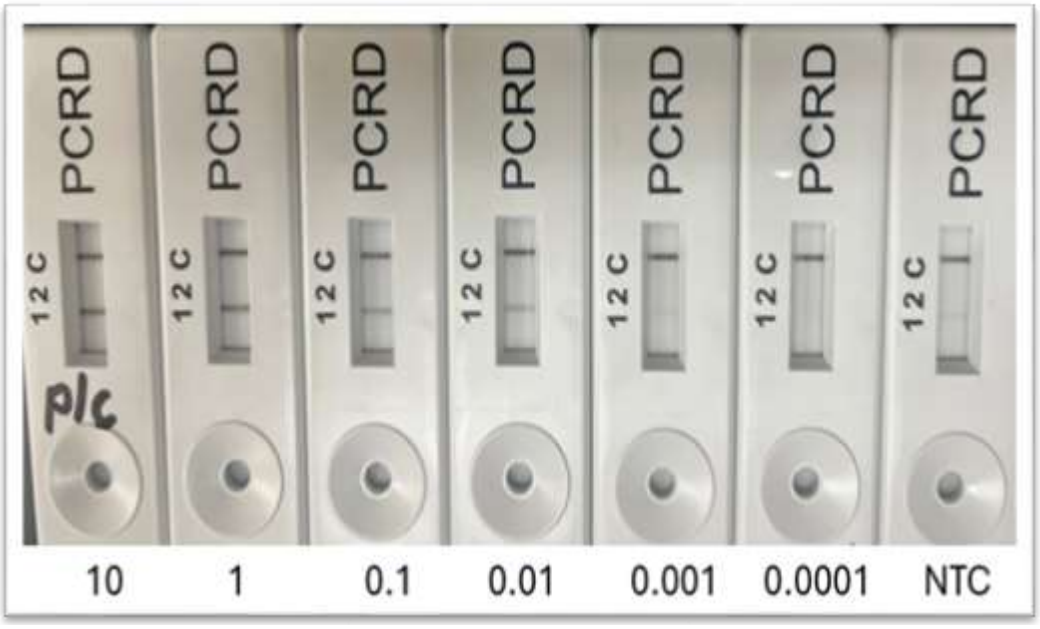

Fig. 3.12. Lateral flow detection of labelled *plc* RPA generated amplicons.

The numbers below the strips indicate the amount of template DNA (in ng); NTC = no template control. This assay had been done at twice; this picture represents one replicate. This test was performed to determine if labelled amplicons were detectable on PCRD device. DNA of 10 ng at a temperature of 37 °C were used for amplification. Test line 1 detected *C. perfringens* dual labelled amplicons.

3.5.6 Testing of the RPA-LFA method using real world samples

Water samples tested are reported in Table 3.3. Samples were quite varied in colour, bioburden, and origin. DNA was extracted using DNA extraction kit 1, as this kit had the least number of steps and is very easy to use, has a special spin column to remove PCR inhibitors (and these can be purchased separately from kit as well), plus to date no reports in South Africa mentioned its use in local water surveillance**.** This is despite it being easily available, despite its ease of use and reported efficiency with various microbes.

As seen in Table 3.3, varied amounts of water were filtered due to differences in bioburden. This was a reflection of the amount of material present in the water; those with higher bioburden clogged filters faster (with less volume of water). This was reflected also on the filters (Fig. 3.13).

TABLE 4. DESCRIPTION OF WATER SAMPLES TESTED IN STUDY.

SAMPLE	APPEARANCE	FILTERED VOLUME
Swimming Pool	Clear	2 l
Tidal Pool	Clear	1 l
Standpipes, Water Tanker	Clear	2 l
River	Slightly murky	200 ml
Potential Reuse H_20	Clear, some speckles of sand	1 l
Wastewater	Very dirty, high bioburden	50 ml
Industrial Waste	Very dirty, high bioburden	2x 50ml

Fig. 3.13. Filtrates of water from water of different origin and bioburden.

Water sample type are as follows: River (A) Swimming pool (B) Tidal pool (C) Industrial effluent (D) Standpipes (E)

DNA from the abovementioned samples were tested first for the presence of bacterial DNA and PCR inhibition did not occur due to residual inhibitors. In this instance, inhibition was just measured as the presence/ absence of amplicons and not measured as number of amplicons (as measured with real-time PCR). In other words, we verified that the DNA was fit for purpose, i.e., is amplifiable, and inhibitors in water was removed sufficiently by the kit. Both DNA, before inhibitor clean-up and DNA subjected to inhibitor clean-up generated the expected 1.5 kb amplicons expected for the 16s rRNA gene (Fig. 3.14). The non-specific amplification seen, is typically observed in complex samples, indicating that one or both of the primers likely bind off-target to non-bacterial DNA. We conclude that kit A suffice to extract amplifiable DNA from the limited water sources used in this study. The chemical composition and other analytes in the water was not provided by the municipality, hence

cannot be reported here. However, future studies should include this data when this kit is properly validated.

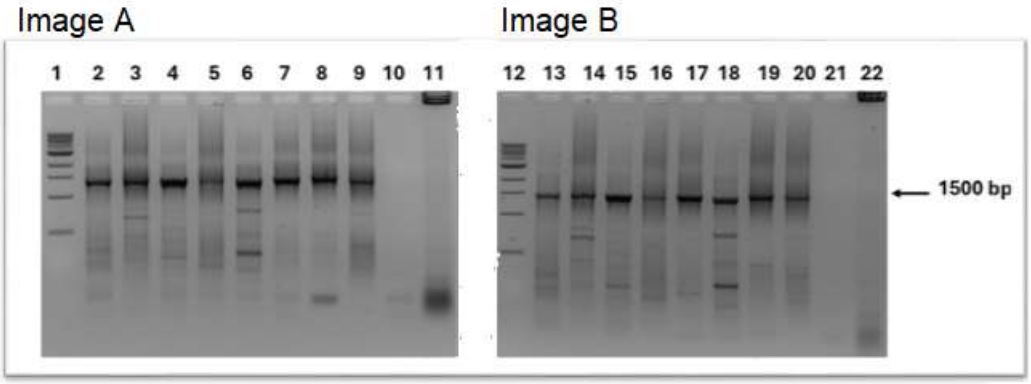

Fig. 3.14. Amplification of 16s rRNA gene using DNA of water origin.

Genomic DNA from water samples were used in PCR, targeting the gene omnipresent in bacteria and a universal marker for bacterial DNA presence. Lanes 1 and 12 – MW marker, Quickload 1KB ladder, Gel on left (image A): PCR done with template prior to inhibitor clean-up (via the provided column). Gel on right (image B), PCR done with DNA template that was subjected to inhibitor clean-up. Amplicons with DNA template: Lanes 2,13, swimming pool, 3;14 – tidal pool; 4,15- standpipes;; 5,16 -river water; 6,17 - water for potential reuse; 7,18 – wastewater, 8,19 – industrial wastewater,9,20 – tapwater;10,21 – negative control- kit reagents put through extraction; 11,22 – negative control, PCR grade water

DNA from selected samples was then subjected to RPA using labelled primers, and amplicons were detected using the PCRD device. As seen in Fig. 3.15, all 3 samples tested were positive for *L. pneumophila* DNA (thus *mipA* positive), while the treated water was only sample positive for *C. perfringens*. Note that the positive control for *mipA* failed on the strip (we did not run gels to confirm amplicon generation though). Also note the faint background for the *C. perfringens* negative control. This was a feature we observed in all RPA generated plc experiments and suggests more optimisation for this primer set is necessary. The same faint result was also seen on the two negative water samples, thus suggesting a background. Also note that none of the amplicons were cleaned up prior to loading on the LFD devices.

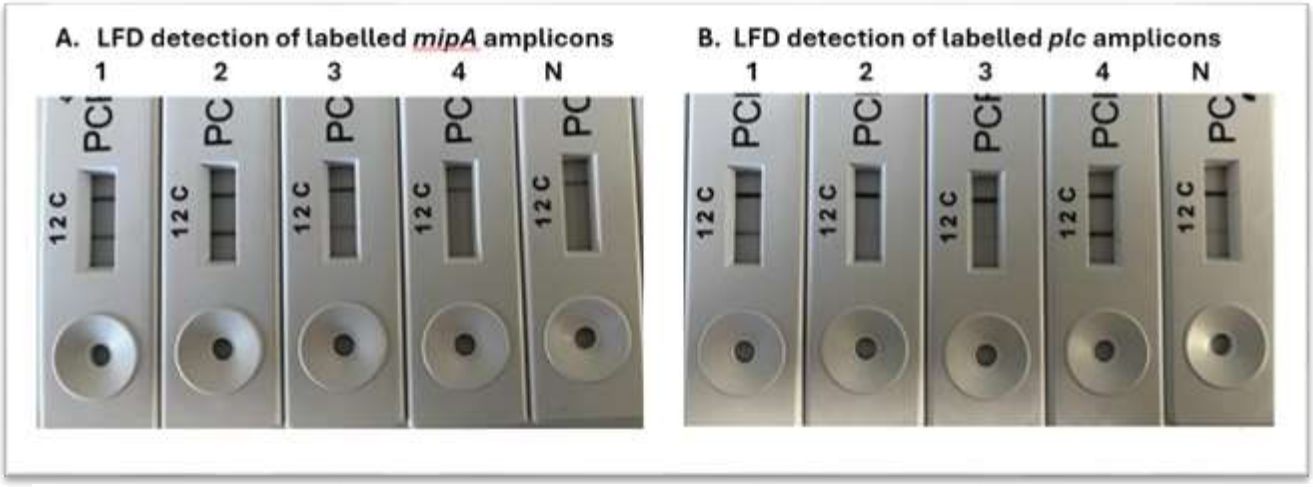

Fig. 3.15. Detection of *C. perfringens* and *L. pneumophila* in DNA purified from water samples.

A – amplicons from *L. pneumophila*; B – amplicons from *C .perfringens*. Samples tested on LFD: 1 – DNA from treated water for possible reuse; 2- DNA from a tidal pool; 3- water from industrial effluent;4 – positive control (DNA from cultured organism); 5- negative control- PCR grade water.

We concluded that the RPA method to detect *C. perfringens* is feasible, generated the correct size amplicon, and labelled amplicons were successfully captured on a lateral flow device. Not that some background was visible on LFD strips (for the negative control), and pools of primer dimers present on RPA gels (not shown) indicates more optimisation is needed for this specific assay. Only one replicate was done for these water samples; future work should include multiple replicates to verify reproducibility etc., especially if samples contain low burden of a target.

CHAPTER 4: DISCUSSION

Molecular identification of pathogens provides a means for rapid identification of pathogens in water. Successful molecular methodology is reliant on the availability of good quality extracted nucleic acids (DNA and RNA). Notably, water contains PCR inhibitors such as fats, proteins, polyphenols, heavy metals (90), polysaccharides, metal ions, humic acid, chlorine and RNases (93,91,94,89,80,92). Another factor impacting DNA quality and quantity is the nature of the microbes and other materials. For example, Gram-positive bacteria cell walls are harder and thicker than Gram-negative bacteria cell walls making it more difficult to lyse (212,213). It is therefore important that DNA extraction methods are optimal for the diverse types of microorganisms. Therefore, in this study, we investigated three different DNA methods to isolate high-quality DNA from two pathogens, with human health implications, in water quality management. As discussed in the literature review, *C. perfringens* has been suggested as an indicator to monitor the efficacy of disinfection and removal of more hardy microorganisms such as *Cryptosporidium*, *Giardia*, helminth eggs and protozoans (56). C*. perfringens* has been linked to human myonecrosis and acute gastrointestinal infections ranging in severity from diarrhoea to necrotizing enterocolitis (214,215). *Legionella spp.* has also been found to be present in water distribution systems, air-conditioning devices, spa baths, and cooling towers (216,22). As *L. pneumophila* is associated with Legionnaire's disease and warm water reservoirs, it is becoming increasingly important to monitor this microbe. The US Environmental Protection Agency (US EPA) has identified *L. pneumophila* as a microorganism of emerging public health concern due to outbreaks associated with various water systems where civilians had been exposed to contaminated "clean" water (217). The resilience of the organism in water systems (such as water pipe infrastructure and also disinfectant exposure) is that this infectious agent can be exist in these structures in a viable but non-culturable state, facilitated by exposure to environmental stressors and disinfection treatments (61). Culturing will likely fail for VBNC organisms, thus making DNA detection technology (such as PCR and isothermal amplification) a more suitable technique for detection of *Legionella*. These techniques are also more sensitive as it can detect very little DNA present in a system, whereas culturing might generate false negatives (218).

Detection of amplicons generated via conventional PCR and RPA.

For this study, the genes to target were selected based on its specificity to *C. perfringens* and *L. pneumophila*, having been evaluated by others in PCRs and isothermal applications to detect these organisms. While phospholipase genes are not unique only to *C. perfringens,*

the nucleotide sequence is sufficiently different from other bacteria also encoding for phospholipase C (67,219–223). For *Legionella,* the *mip* gene is unique to *L. pneumophila* although *mip*-like genes are found in other species (224). Nevertheless, this gene had been used by various authors in real-time and isothermal assays (152,158,225). Notably, the *mipA* gene identifies any *L. pneumophila* present in a sample, and is not unique to Serogroup 1, which is responsible for most cases of disease (225–227).

We first evaluated the *mipA* assay, using previously published primers and RPA conditions. New primers were designed for *C. perfringens* plc, checked *in silico* and via conventional PCR plus Sanger sequencings, validating it generate the correct amplicons. In our *plc* assay, within the limits of this MSc study, only time-to-positivity and temperature were evaluated, not dNTP concentration. Initial RPA was done using 20-30 ng of DNA, to check amplicon generation and get products for Sanger sequencing. Products were not cleaned up prior to detection on gels and LFD devices. An optimised assay that does not require clean-up (minimal or no background) is ideal for fieldwork as clean-up usually is done via columns and requires an electrified device (which might/might not be available). The dNTP concentration was not titrated in this study as the lyophilised kit already contains dNTPs, meaning only more dNTPs can be added to the reaction (not necessary as the assay worked and other factors must rather be optimised). Future studies therefore, could look at primer optimisation, either design of improved primers or using fewer primers in assays to avoid primer dimer formation.

For field work and to do RPA using body temperature (approximately 37 °C), an assay optimised at this temperature is ideal as it would exclude the use of a heated instrument such as a heating block or PCR instrument (shown already by others). Therefore, optimisation of primers is the most likely best way forward, if one wants to design an assay that works at body temperature (or a small mobile device heated by the body as shown in literature (137,138). Ideally an appropriately low primer concentration and best primer pair are needed that will not give any background on lateral flow devices.

In this study, we also evaluated if dual labelled amplicons were successfully detected using a commercial lateral flow device that contained antibodies to capture DIG or 5-FAM labelled amplicons on one end and labelled with biotin on the other end. We used a commercially available lateral flow device. We demonstrated that labelled mipA and plc amplicons generated via both conventional PCR and RPA were successfully captured (Fig 3.5, 3.7, 3.11, 3.12), suggesting both methods potentially could be useful for surveillance with properly validated assays. The method for *plc* requires further validation though, using DNA

extracted from a greater variety of real-world water samples, and the true limit of detection should be determined with these real-world (as these might have different inhibiting factors influencing PCR outcomes).

When comparing agarose gel detection vs LFD detection, *mipA* was detected using as little as 1 pg of input DNA. Detection on the LFD detection provided better, even though there was a very faint band when visualised with UV transillumination on the agarose gels. This implies improved detection sensitivity via LFD, but we have not done sufficient replicates and need to be repeated in future studies to verify this observation. Also, the *mipA* RPA sensitivity had been tested and reported in the literature by Kerstinga and co-workers (158) who developed this *L. pneumophila* RPA assay (primers designed by Kerstinga et al.,(2018) (158) were used in this study). Thus, evaluation needs to be done with water samples, if they are used for surveillance in water.

We observed some smearing in our agarose gels and some faint backgrounds on LFDs. RPA amplicons are typically purified via column clean-up to remove unincorporated reagents such as primers and primer-dimers or low level non-specific amplicons. It was noted by us (and others) that the proteins and the crowding agent present in the amplification mix can result in the smeared bands (228). As indicated, the RPA amplicon gels in this study showed the presence of non-specific smearing and very faint, large fluorescent band and primer dimers when the *plc* amplicon was absent (Fig. 3.6, 3.9 and 3.10). Notably, many RPA published papers report cleaned up products ran on agarose gels (229,230). As reviewed by Daher et al., (231), RPA amplicons need to be cleaned up prior to loading on gels, for correct interpretation and exclusion of false positives. In this study however, neither *plc* nor *mipA* amplicons were cleaned up, as we wanted to discern the presence of non-specific bands and primer dimers; this is necessary so that one can then appropriately optimise the assay specifically with the relevant parameters necessary. In the Kerstinga *mipA* study (158) , the authors also noted some false positives (faint detection) when using only labelled amplicons and thus modified their reported assay by adding of a probe to make it more specific. In the reported assay, as little as 10 CFUs of the organism yielded a signal via lateral flow detection. The genome of the organism is approximately 3.4 Mb and the authors calculated that 2.72×10^5 colonies would yield approximately one nanogram of DNA; thus detecting 10 colonies is very sensitive (picogram amounts of DNA detected). The assay using the probe is thus very sensitive (using both labelled amplicons plus a probe). In our study, we did not use a probe and just wanted to verify that the assay worked in our hands (proficiency) before proceeding to develop another

assay for a different organism; we show successful detection, and also observed very faint potential false positives. To reiterate, validation of LOD needs to be done with actual real world samples, as the value observed with spiked samples or pure cultures might shift, based on the origin of the DNA, the bioburden and how devoid DNA is of PCR inhibitors (such as humic acid and other potential chemical content not removed during DNA extractions). Notably, in water with industrial effluents (which might vary), DNA extractions might be trickier, depending on the type of pollutants present.

Looking towards the published literature on RPA, studies conducted by Sun et al., (232) concluded that the test line become difficult to visualize when DNA extracted from approximately $1x10^5$ cfu/ml *L. pneumophila* cells were used (232). Another study successfully detected as little as 10^1 colonies/ml of this organism (152). In our study with *L. pneumophila,* we observed that DNA of 10 ng and 1 ng yields amplicons that are clearly visible on the LFA strip. Studies conducted by Stone et al., (24) successfully detected *L. pneumophila* from domestic water heating systems using conventional PCR and qPCR, reporting similar sensitivity. Nevertheless, our study showed that the organism will likely be detected in water systems using isothermal methods such as RPA, although we only tested three water samples for LFD detection (Fig. 3.15). Future studies should expand to a variety of water samples, including water from systems known to be polluted by biofilms. Furthermore, as *L. pneumophila* is known to live intracellularly inside various Protists (see Literature review), DNA extraction methods need to be optimised to ensure bacterial DNA is extracted as both Protists and bacteria need to be lysed. In this regard, extraction kits or methods needs to be validated for sufficient lysis of Protists found in water systems.

For *C. perfringens, DNA* input of 10 pg and 1 pg yields PCR amplicons that were detectable on the LFD. The positive control was faint, however, in gels (Fig.3.10, lane 9) and the LFD, potentially as this DNA was from an older extraction, and had been freeze/thawed multiple times to use for positive controls. Also, the dual labelled *plc* amplicons did not provide strong signals like the *L. pneumophila* amplicons, suggesting that potentially fewer amplicons are generated for this *C. perfringens* RPA and thus implies more optimisation is needed. We searched online literature for the RPA detection of *C. perfringens* in water using conventional PCR or RPA and lateral flow strip; these were not found in English-language publications. However, a successful study conducted by Sridapan et al., (233) incorporated Lateral flow strip and LAMP technology (another isothermal method) to detect *C. perfringens* in food (233). In this LAMP study, also targeting the phospholipase C gene, the authors reported 10 ng/µl of DNA (or 1 cfu/ml of the organism) as the lower LOD. Although the method is

different to RPA, the outcome is similar to the lowest amount of input DNA detected via our RPA assay. Nevertheless, our observations need to be done with sufficient replicates of pure cultured material, spiked material as well as real world samples.

Regarding the RPA kit used: The initial RPA optimisation for *C. perfringens* was attempted with the TwistAmp Liquid Basic kit. As non-specific amplification was seen at higher temperatures, the outcome demonstrated that 37 °C was suitable for amplifying the *C. perfringens plc* gene and the *L. pneumophila mipA* gene fragment. However, further work was done using the lyophilised Basic Kit as this was much easier to set up, had fewer steps and was more amenable to point-of-care work. We also did not observe the non-specific, large band(s)/smear when using this kit (see Fig 3.8, reporting amplicons done using the lyophilised kit compared to Fig 3.9 (showing an example of RPA with the liquid Basic kit; the gel for amplicons with the liquid kit and time titration not shown, but has similar non-specific smearing as Fig 3.9). We also did not observe the non-specific, large band(s)/smear when using the lyophilised kit. We therefore conclude that the lyophilised kit might be the better option for further assay development, notably as it is much easier to set up.

The study reported here used purified bacterial cultures and was able to detect as low as 1 pg input DNA when amplification was done for 30 minutes at 37 °C. Future studies could do more time titration studies, including with water samples to ascertain how low and high bioburden, varied pollutants etc. could impact amplification efficiency and time; this of course also depends on the extraction kits used and the availability of reagents to remove PCR inhibitors (not all kits contain these).

Comparison of DNA extraction kits

An important aspect of successful molecular detection is the availability of sufficient target material and the absence of PCR inhibitors or the presence of negligible concentrations thereof. Various studies extracting DNA from water or from waterborne pathogens have been used, using a variety of DNA extraction kits. For example studies conducted by Hoorzook and Barnard 2022 used the Water Master™ DNA purification kit Ultra Clean™ Water DNA isolation kit, Aquadien™ kit, Metagenomic DNA isolation kit and in-house guanidinium thiocyanate DNA extraction method to extract DNA from water samples(234). The study concluded that the in-house guanidinium thiocyanate DNA extraction method was a better cost alternative with good recovery, repeatable and reproducible results. Unfortunately, in-house methods are more time-consuming and generate chemical waste that are not environmentally friendly and additional costs to safely

dispose of. The FastDNA® Spin Kit for Soil (MP Biomedicals), PowerSoil® DNA Isolation Kit (Qiagen) and ZR Fecal DNA Miniprep kit (Zymo) are other popular, spin column-based kits used in various reported studies on wastewater and freshwater sediments (191,192). Studies conducted by Li et al., (191) using the FastDNA® Spin Kit, PowerSoil® DNA Isolation Kit and ZR Feacal DNA Miniprep kit on influent, activated sludge and effluent samples for the enumeration of antibiotic resistance genes. They concluded that when comparing influent, activated sludge, and effluent samples taken from two WWTPs situated on different sides of the world, the three DNA extraction kits revealed comparable patterns. Nonetheless, the FastDNA Spin Kit for Soil performed better across the board for all sample locations (Hong Kong and USA wastewater treatment plants) when yield, quality, diversity of ARGs and taxa from the extracted gDNA, and reproducibility of the extraction were taken into account (191). Another study conducted by Shi et al*.,* (192) extracted gDNA from freshwater sediment utilizing the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), and MinkaGene Soil DNA Kit (Magigene Biotechnology, Shenzhen, China). The extracted gDNA was evaluated in terms of DNA yield, quality, and reproducibility. More eukaryotic operational taxonomic units were identified, and a significantly greater DNA yield was generated with good consistency using the FastDNA SPIN Kit for Soil and the DNeasy PowerSoil Kit. All three kits showed a similar capacity to recover the variety of bacteria with alpha diversity (192).

These spin column kits are easy to use and generally are reported to yield good quality, fit for purpose DNA. Another report of a local study used the QIAmp DNA Blood Mini Kit to extract DNA of *L. pneumophila* from a hospital water network in Johannesburg. Of the 98 water and biofilm samples gathered, 71 (72.4%) contained free-living amoebae. On BCYE and GVCP agar, Legionella did not grow from the amoebae-positive samples. However 9.9% of the samples were positive for *L. pneumophila* for both conventional PCR and qPCR. This is most likely due to the Amoeba-resistant *L. pneumophila* remnants that underwent a VBNC condition are probably still there (18). A major benefit of manual commercial DNA isolation kits is that they produce findings faster (compared to phenol: chloroform and desalting based in-house methods) and require fewer processing steps. A significant drawback, however, is that commercial miniprep kits limits the volume of samples to process, columns tend to get blocked if too much material is present. These kits generally also yield less DNA than the in-house, chemical purification methods. While not explored in this study, this has implications for water samples containing different bioburden and addition chemicals. The volume of samples needs to be optimised and validated to ensure a specific selected kit yields optimal, reliable, reproducible results. This is an essential step

laboratories and researchers need to do before embarking on a particular method.

Therefore, DNA extraction methods must be validated for the quality and quantity to predict downstream usefulness (for PCR, Next Generation Sequencing etc.). As mentioned previously, the ratio of absorbance at 280 nm and 260 nm is used to determine the purity of the DNA; a value of 1.8-2 is considered pure DNA. A value obtained less than 1.8 is an indication that the DNA extract is not pure and may contain contamination such as proteins (205). A ratio of absorbance at 260 nm and 230 nm is used to detect unwanted organic compounds, generally from the extraction process. Values of 1.8 and 2 typically indicate purer DNA(235). However, impure, crude DNA extracts (such as from boiled bacterial cells are fit for PCR, except if the matrix it originates from contains PCR inhibitors. Therefore, for this study, the DNA extracted from the pure, cultured cells should be fit for PCR despite the values outside of the 1.8-2 range.

For all three extraction kits, purified *C. perfringens* DNA yielded absorbance ratios below 1.8 at 260/230 nm (Table 3.1). Ratios below 1.8 are often result of contamination from contaminants such as proteins, guanidine HCL, EDTA, carbohydrates, lipids, salts, or phenol, which have an absorption ratio of 230 nm (236). These low ratios are not an indication that PCR amplification will be inhibited, yet DNA from real world samples that might contain varied chemical contaminants must be evaluated to determine if it is fit for purpose.

Table 4.1 indicates a list of reagents from the tested kits which might be found in small quantities in the final extracted DNA solution and could inadvertently influence its purity and result in PCR inhibition.

TABLE 5. POST EXTRACTION, POTENTIAL RESIDUAL CONTAMINANTS FROM COMMERCIAL KITS

Kit	Proteins	Guanidine	Salts
Quick-DNA Fungal/Bacteria Miniprep kit	Negligible	Negligible, Guanidinium thiocyanate Guanidinium chloride	Edetate Disodium, Dihydrate
Mag-bind® Environmental DNA 96 kit	Not known		Sodium Azide
Wizard® Genomic DNA	Protein precipitation solution	Not applicable	Not applicable

Likewise, the absorbance at 260/280 nm for all three kits gave values below 1.8 with except for two extracts giving ratios of 2.19 and 2.39 when the Mag-bind® Environmental DNA 96 kit was used (Table 3.1).

Gema Lucena-Aguilar et al., 2016 (236) indicated that the 260/230 ratio could be unreliable in cases where salt based elution buffers are used to dissolve the DNA. In the case of the Quick-DNA Fungal/Bacteria Miniprep kit and Mag-bind ® DNA 96 kit, both kits contain salt, and the 260/230 results were low.

The Wizard® Genomic DNA Purification DNA purification kit generated the highest concentration of DNA compared to the other two kits (Table 3.1). As the same number of bacterial cells were used for the three extraction methods, this indicate that some nucleic acids are lost using the column or magnetic bead methods or could also be an indication of residual RNA in the Wizard kit method. However, it should be that the DNA extraction comparison study was conducted using pure cultures. Water samples may contain inhibitory substances that could further influence the extraction outcomes and methods, based on the bioburden and complexity of samples. Further studies should include the testing of water samples using various DNA extraction kits, to verify which kits are fit for purpose (and if they are one-size-fits-all).

Comparison of the DNA extraction kit with regards to amounts of steps, time to obtain pure DNA and equipment needed (Table 3.2) the Quick-DNA Fungal/Bacteria Miniprep kit and Mag-bind® Environmental DNA 96 kit utilizes a mechanical cell lysis step (bead beating) whereas the Wizard® Genomic DNA kit uses an enzymatic lysis method. It is a known fact that mechanical cell lysis improves DNA yield (237–239), but its drawback is that more DNA shearing can occur, resulting in lower DNA yields, and/or degraded DNA (240–243). Also,

soil and sediments contain humic acids, which can be extracted during bead bashing (244,245). Potentially, therefore, a bead beating step might not be appropriate for humic acid naturally containing soil sediments and alternatives such as more efficient enzyme digestion of cell envelopes should be explored. For example, in a review paper (not yet peer reviewed, on BioArxiv), Rieder et al., 2023, suggest that chemical lysis methods preserve DNA integrity better in comparison to mechanical lysis. However chemical lysis methods require thorough clean-up, ideally multiple clean-up steps, following the use of organic solvent to prevent negative effects on downstream enzymatic procedures. The paper further indicates that enzymatic digestion with lysozymes, ribonucleases, proteases and proteinase K, are more gentle methods that preserve the integrity of DNA (242).

However, a comparative study conducted by Gill et al., (2016) on vaginal microbiota concluded that lysis using lysozyme, in conjunction with a bead-beating step, resulted in noticeably lower yields than either lysis using the enzyme cocktail (containing lysozyme, mutanolysin and lysostaphin) or lysozyme alone for 30 minutes (241). Studies also conducted by Teng et al., on oral microbiota indicated that higher amounts of DNA were obtained when enzymatic lysis was used in comparison to the bead beating or bead with lytic enzyme combination (246). Enzymatic lysis, via enzymes such as lysozyme, dissolves the bacterial rigid cell walls (247) or cell walls containing peptidoglycan. This process may be especially significant for the breakdown of the thick peptidoglycan layer found in Gram-positive cell walls (241). It is worth noting that *C. perfringens*, is a Gram-positive bacterium, which contains peptidoglycan in its cell walls, as well as a spore form that is even harder to obtain DNA from (248) and was used in this comparative study of the DNA extraction kits. Future studies should investigate the potential of these enzymes for lysis of *C. perfringens* and *L. pneumophila*. A significant problem is to obtain DNA from Clostridial spores. The hardy cell wall presents an additional barrier to obtain good DNA.

One report on *C. tyrobutyricum* spores explored various methods to optimise DNA extractions from spores in contaminated milk. It was found that microwaving (15 min at 600 W) of spores prior to extraction yielded more DNA compared to 15 min at 600 W (249).

A significant consideration in kit selection for diagnostic use, especially at point of care, is the time and number of steps needed for extraction. As indicated in Table 3.2, the Mag-bind® Environmental DNA 96 kit has 39 steps, significantly more than the Quick-DNA fungal/Bacterial kit which is ten steps and the Wizard® Genomic DNA Purification kit which is 12 steps. The number of steps required thus can increase the risk for technical errors such as pipetting errors, switching of samples and cross-contamination. Another

consideration is the presence of residual chemicals in extracted DNA; these could interfere with PCR. For example, the Wizard® Genomic DNA Purification kit requires ethanol and isopropanol for the washing steps; these are known PCR inhibitors (91,250). These can be removed by so-called PCR inhibitor removal steps. As mentioned previously, two out of the three kits contain PCR inhibitor removal steps. However, the Wizard® Genomic DNA kit does not contain a PCR inhibitor removal step which can result in interference during the amplification process (251) even though the yield of DNA is greater. However, alcohol is required to precipitate the DNA, and therefore the technical person must ensure that all alcohol has evaporated before DNA is reconstituted. This is time-consuming though, especially if DNA yield is high. Another consideration against kits requiring salt precipitation and reconstitution of DNA Is that it be noted that the risk with these steps is the loss of partial or complete cell pellets.

With regards to DNA yield, the Quick-DNA Fungal/Bacteria Miniprep kit did not yield as much DNA as the Wizard® Genomic DNA (Table 10)However the kit contains an additional spin column that removes the inhibitors (89,252); an advantage for DNA extracted from water with potential inhibitors. Furthermore, these specific columns can be purchased separately, thus extracted DNA from any platform could potentially be cleaned up from inhibitors using this column. Furthermore, the kit has fewer steps, thus less time consuming and reducing the risk of analyst errors, in comparison to the Wizard® Genomic DNA Purification kit and Mag-bind® Environmental DNA 96 kit. Importantly these kits are not commonly available in South Africa, adding to an increase in cost due to import levies and shipping fees, as well as possible delays at customs.

In terms of infrastructure and equipment required, unfortunately, all three kits require the use of electricity, thus not ideal for point of care setting. Ideally, methods generating PCR-able DNA generated via methods requiring minimal electrical equipment or no purification is ideal for POC testing. Chemical lysis techniques, for instance, are straightforward and do not require a lot of equipment. Mechanical lysis techniques are chemical-free but necessitate the use of additional equipment. Furthermore, chemical lysis chemicals can potentially to impede downstream bioanalytical detection techniques like PCR and membrane sensor comparison (253).

With the above mentioned pros and cons in mind, it is clear that the ideal DNA extraction kit (for POC use) should contain cell wall degrading enzymes functioning at room temperature (thus excluding the use of an electrified heated incubation step), plus effective inactivation and removal of enzymes and residual chemicals. These incubation steps ideally should

exclude electrified equipment. It is thus interesting that so called extraction-free PCR solution, using crude DNA (not purified, from boiled microbial material) are available (254). However, extraction free methods most likely would not be applicable for complex, high bioburden water samples. It is unlikely that amplifying of crude DNA from boiled complex water samples contaminated with PCR inhibitors would be fit for purpose, i.e. not amplifiable. In light of this, extraction kits should be evaluated for its ability to extract fit for purpose DNA from varied complex matrices and these ideally should need minimal electrified equipment.

Future studies should:

Investigate extraction free methods with different water sources, although extraction free methods would be more applicable for samples such as repurposed water (example is treated water used for irrigation), or seawater used for municipal swimming pools etc.

Compare locally available kits using water samples of differing complexity, to verify specific kits are extracting quality DNA from all sample types and other magnetic bead methods and kits on the market (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9804433/), as magnetic bead provides a quick form of DNA purification, with limited use of environmentally unfriendly chemicals. However, magnetic beads themselves, needs to be disposed of appropriately, so laboratories must investigate local guidelines on magnetic bead disposal.

The use of live-dead microbial staining dyes (PMA, EMA) incorporated into RPA amplification methods must be investigated in order to distinguish viable, residual pathogens in tested samples. Testing should include verification that these dyes penetrate the bacteria envelopes of targeted microbes.

CHAPTER 5: CONCLUSION

In this study, we investigated RPA as an isothermal DNA amplification technique useful to amplify bacterial pathogen DNA, using *C. perfringens* and *L. pneumophila*. The rationale for selecting these pathogens was that clostridial spores (and vegetative) cells, as well as intra-cellular living *L. pneumophila*, are more resilient to chemical disinfection processes and, thus, high-risk pathogens that could escape water treatment processes. Furthermore, *L. pneumophila* is also is known to prevail in water infrastructure of buildings and geysers, mostly found intracellularly inside protozoans. Using pure, cultured reference isolates, DNA was extracted and successfully amplified using a commercially available RPA kit designed by TwistDX. A previously published RPA primer set for *L. pneumophila*, was used and shown to successfully amplify *L. pneumophila* DNA, replicating the published RPA protocol. Using in-house designed primers for the *C. perfringens* phospholipase (*plc*) gene, we successfully optimised this RPA assay targeting this microbe. Further development is necessary when testing with different water matrices. However we demonstrated that the correct target is amplified and verified using DNA sequencing.

Secondly, we used labelled primers to generate dual labelled amplicons (using DIG and Biotin as labels) that can be captured on a commercially available lateral flow device embedded with antibodies against DIG and Biotin. Using this approach, we demonstrated that a workflow using RPA (to generate dual labelled amplicons via an isothermal amplification method) combined with lateral flow detection could be a solution to detect labelled amplicons generated from targeted microorganisms. Both assays, against the targeted microbes in this study, detected amplicons generated from as little as picogram amounts of input DNA. This level of sensitivity might change when using DNA that is extracted from complex water matrices, as different matrices might contain different PCR inhibitors. Thus, laboratories using this methodology, must test it and optimise it using water of differing origin or complexity.

Using a limited number of water samples, representing low to high bioburden, as well as differing assumed chemical composition, we demonstrated that the assays detected *C. perfringens* and *L. pneumophila* DNA in the tested water matrices using this RPA generated, dual-labelled-LFA detection system.

A gap in this study is that bacterial viability testing was not done. Culturing itself is also not ideal as bacteria can be present in a viable but non-culturable form in aqueous environments. Future studies should look at developing DNA amplification methods that

incorporate viability confirmation in the actual PCR (so-called viability PCR). This was not in the scope of this study, where we aimed to test an isothermal method not currently used locally in diagnostic settings.

Lastly, DNA extractions are a limiting step in diagnostic testing. While it is ideal to have extraction free molecular surveillance methods, this is not ideal for water testing as inhibitors in samples interferes with amplification methods. Therefore, commercial kits, or in-house extractions methods will probably remain a key step in a molecular workflow to do surveillance in water. This would be especially important for water with heavy contamination of humic acid, mammalian debris, and heavy chemical pollution, as potable or reused water would only contain residual chlorine, which is easily removed via neutralisation with sodium thiosulphate. While certain commercial kits are more readily used by water researchers, few have done comparisons between kits using different modes of DNA purification. In this study, while not testing actual water samples, we used cultured *C. perfringens* to evaluate three kits that are locally available, each representing a different mode of purification. Generally, it is more difficult to extract DNA from this microorganism (compared to coliforms or other water pathogens), hence our choice of microorganism to test. Secondly, this microorganism is a major concern as a potential residual pathogen in water intended for reuse, as it is more resilient against chemical disinfection. Using a minimum of 10^6 bacterial cells and eluting in less buffer (40 µl vs recommended 100-200 µl) than recommended by the different kits, the tested kits generated sufficient DNA to be detected and quantitated using UV spectrophotometry. This is an important consideration when extracting DNA from water with differing bioburden as more cellular material will impact the DNA extraction methods and elution volumes. Laboratories should consider this when validating DNA extraction kits to be used for diagnostics. DNA from all three kits were amplifiable (tested using conventional PCR), although the purity differed between the three kits. A cost, infrastructure needed, and ease of use comparison was also done, indicating that the spin column based clean-up method was easiest to use, while the magnetic bead kit had too many steps in the procedure and thus was lengthy. At the same time, the chemical clean-up kit was very simple and efficient and yielded higher amounts of DNA, yet the DNA was of lower purity compared to the spin column and magnetic bead-based kits. There was no real difference in terms of the cost of the kits, and all easily available on the local market (no need for direct import). The kits investigated in this study had not been reported in publications from local researchers doing water microbial monitoring, although two of the kits (spin column and magnetic bead-based) both have

steps to remove PCR inhibitors found in environmental samples (water, soil, faecal matter). These would thus be good choices to investigate for diagnostic use.

In conclusion, this study presented an alternative to conventional and real-time PCR that requires expensive instrumentation and a reliable source of electricity. While not evaluated in this study, the literature indicates that RPA amplifies DNA even at room temperature or body heat. It should thus be investigated further to test amplification using heating blocks and body temperature. The use of either of these forms of heating would exclude the use of PCR instruments. Future studies should expand testing using water matrices of different bioburden and chemical complexity and appropriate DNA extraction methods related to the water matrices to be tested.

APPENDIX 1. MEDIA

Preparation of agarose gels

To prepare agarose gels, 1 g of agarose powder was added into 100ml of 1X TAE, generating 1% gels respectively. The solution was boiled in a microwave to dissolve the agarose powder. Three microliters (3 µl) of ethidium bromide per 100ml of TAE was added prior to solidifying in order to visualize the DNA post-electrophoresis using the Gel Doc ™ XR imaging system (Bio-Rad).

50x TAE was purchased from Greenpoint Media (NHLS, Cape Town).

APPENDIX 2. FBC APPROVAL LETTER

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences

FACULTY RESEARCH ETHICS OFFICE

Private Bag X3, Rondebosch, 7701, South Africa
Barnard Fuller Building, Anzio Road, Observatory, Cape Town
Tel: +27 (0) 21 650 5057 or 6087 Fax: +27 (0) 21 447-8955
E-mail: sidney.engelbrecht@uct.ac.za

FACULTY BIOSAFETY COMMITTEE (FBC)

15 September 2021

FBC REF.: LP/004a/2021-2024

Dr L. Paul
Division of Medical Microbiology
Department of Pathology
Faculty of Health Sciences
University of Cape Town

Dear Dr. Paul

Project Title: ***DEVELOPMENT OF ISOTHERMAL PCR-LATERAL FLOW DETECTION METHOD TO DETECT PATHOGENS IN WATER***

Thank you for submitting your response to comments from the Faculty Biosafety Committee (FBC) in the Faculty of Health Sciences.

The response has been reviewed by the FBC and no substantive concerns have been identified.

Therefore, your application is approved, effective dates 15 September 2021-16 September 2024.

You may commence work on this project immediately.

Please note that the application and FBC recommendation will be tabled for noting at the next meeting of the University's Institutional Biosafety Committee (IBC).

Yours Sincerely

Professor Digby F. Warner
Chair: Faculty Biosafety Committee

APPENDIX 3. TURNITIN REPORT

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