‘PREGNANCY TEST’ FOR WATER
EXPLORING THE USE OF IMMUNOASSAYS TO DETECT BACTERIAL PATHOGENS IN WATER

Narrative report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

BACKGROUND

Imagine if testing water for the presence of diarrhoea causing bacteria was as easy as doing a pregnancy test at home. The appearance of a simple red line could indicate if the water is safe to consume, and if not, could possibly indicate what the contaminating bacteria is. If we could even do it in less than 18 hours (the standard time for accepted microbiological tests) we could rapidly adapt our water treatment process and patient's treatment protocols.

Even today, water testing is geared towards the presence or absence of indicator organisms that indicate the possible presence of other disease-causing microorganisms. These tests typically take between 24-48 hours to complete before we could start "searching" for the disease-causing microorganism in the water. To speed up the process scientists have started using molecular biology methods to detect and describe the DNA from the bacterial pathogens in water, but this is still typically confined to laboratories that have the equipment and expertise to run the experiments. More importantly these tests would still be done on bacteria already isolated from the water.

If we want to develop a test that could theoretically be run in the field without a laboratory, we need to make sure that we can remove as much of the laboratory equipment from the equation. This would include ways to recover the bacteria from the water, how to detect the bacteria without first growing them in the laboratory and lastly how to confirm the type of bacteria present in the water.

RATIONALE

In our Centre we already have a homemade method that can concentrate the bacteria from the water and break the bacterial cells open to access the DNA. The remaining steps are to be able to screen, and visualise, the DNA for the presence of bacteria specific genes, much like any **C**rime **S**cene **I**nvestigator (CSI) that uses DNA to track criminals. The screening process, called **P**olymerase **C**hain **R**eaction (PCR), allows for the multiplication of the specific genes from the bacteria, and this process still requires some equipment. It must be mentioned that the affordability of this equipment has increased making it more accessible for use. The remaining step is the visualization of the DNA to determine if, and possibly what, bacteria is responsible for the illness.

The visualization of the DNA was the goal of this project and specifically focused on how we could use the science used for the pregnancy test to develop a visualization tool for the presence of bacterial DNA.

METHODOLOGY USED

To achieve this goal, we developed a “multiplex lateral flow immunoassay”, basically a “pregnancy test” (lateral flow immunoassay) that produces more than one red line (multiple) to show the presence of a maximum of three bacterial pathogens per test. The basis for the test is that each gene is linked to a specific dye during the PCR process, and that these dyes then bind to antibodies developed specifically for them to collect them on a specific point on the “pregnancy test” strip. We then add colloidal gold that binds to the collected DNA creating the red line that you would typically look out for.

We used this method to test samples obtained from water, flies, food and dishcloths for the presence of diarrhoea causing *Salmonella*, *Shigella* and *Vibrio* bacteria. Specific strains of these bacteria are responsible for diarrhoea in humans and can be spread through the use or consumption of contaminated water.

RESULTS AND DISCUSSION

The developed test could successfully detect the DNA from the three bacteria at the correct binding sites of the test strips. The method proved to be very specific, sensitive, precise and accurate when detecting the three bacteria in the laboratory. The testing of the method on water, flies, food and dishcloths produced promising results but did show that more work is needed before it can be tested in the field.

CONCLUSIONS

We were able to show that the method can be used to detect and visualise bacterial DNA from samples. It must be noted that this is a drastic over-simplification of the process but conveys the basic concepts and steps for easier understanding. The sections to follow will have more detail to allow for an in-depth understanding of the work.

RECOMMENDATIONS FOR FUTURE RESEARCH

Despite the promising results produced during this study, shortfalls in the method were identified that need to be addressed. The future work will entail improvements in the technology before we start the testing under field conditions.

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LIST OF ABBREVIATIONS

A/E	Attaching and effacing
Ab	Antibody
AF	Alexa-fluor 405
APW	Alkaline peptone water
AR	Acid resistance
ATCC	American type culture collection
$AuCl_3$	Gold (III) Chloride
BO	Bodipy®FL
bp	Base pairs
BPW	Buffered peptone water
BS	Bismuth Sulphite
BSA	Bovine serum albumin
CDC	Centre for Disease Control
Cfu	Colony forming units
cGMP	Cyclic guanosine monophosphate
cm	Centimetre
ComEC	Commensal *E. coli*
CT	Cholera toxin
Ctx	Cholera toxin gene
DCA	Deoxycholate citrate agar
DEC	Diarrhoeagenic *E. coli*
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphates
DWEA	Department of Water and Environmental Affairs
EaeA	*E. coli* attaching and effacing
EAEC	Enteroaggregative *E. coli*
EAST	Enteroaggregative shiga toxin
ECA	Enterobacterial common antigen
ECL	Electrochemiluminescence
EDTA	Ethylene diamine tetraacetate
EHEC	Enterohaemorrhagic *E. coli*
EIA	Enzyme immunoassay
EIEC	Enteroinvasive *E. coli*
ELFA	Enzyme-linked fluorescent assay

ELISA	Enzyme linked immunosorbent assay
EMB	Eosin methylene blue
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
F	Fluorescein
g	Grams
GE	Gel electrophoresis
GF	Glass fibre
Gm-PCR	Genus specific multiplex PCR
GuSCN	Guanidinium thiocyanate
hCG	Human chorionic gonadotrophin
HIV	Human immunodeficiency virus
HPA	Health Protection Agency
HUS	Haemolytic uremic syndrome
H_2S	Hydrogen sulphide
Ial	Invasion associated locus
IBS	Irritable bowel syndrome
ICG	Immunochromatographic
ICH	International Convention on Harmonisation
ID	Infectious dose
IgG	Immunoglobulin G
IMS	Immunomagnetic separation
Ipa	Invasion plasmid antigens
IpaH	Invasion plasmid antigen H
IUPAC	International Union of Pure and Applied Chemistry
KIA	Kligler iron agar
L	Labelled
LCR	Ligase chain reaction
LFIA	Lateral flow immunoassay
LIA	Line immunoassay
LOD	Limit of detection
LT	Heat labile entero-toxin
M	Molar
mdh	Malate dehydrogenase
mg	Milligram
mins	Minutes
ml	Millilitre

mm	Millimetre
mM	Millimolar
m-PCR	Multiplex PCR
NaCl	Sodium chloride
NALFIA	Nucleic acid lateral flow immunoassay
NASBA	Nucleic acid-based sequence analysis
NATs	Nucleic acid tests
NC	Nitrocellulose
NCTC	National Collection of Type Cultures
n.d	No date
NHLS	National Health Laboratory Services
Nm	Nanometre
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pet	Plasmid encoded toxin
PFGE	Pulse field gel electrophoresis
pInv	Invasion plasmid
pmol	Picomole
POC	Point of care
POCT	Point of care tests
PVDF	Polyvinylidene fluoride
Q-PCR	Quantitative PCR
RIA	Radio immunoassay
RIBA	Recombinant immunoblot assay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Real time PCR
SAMs	Self-assembled monolayers
SANS	South African National Standards
SCG	Streptavidin-colloidal gold
secs	Seconds
ShET1	*Shigella* enterotoxin 1
SPI	*Salmonella* pathogenicity island
Spp.	Species
ST	Heat stable enterotoxin
Stx 1	Shiga toxin 1
Stx 2	Shiga toxin 2

SSC	Sodium, sodium citrate
TAE	Tris base, acetic acid and EDTA
TBS	Tris-buffered saline
TCBS	Thiosulphate citrate bile-salts sucrose
TCP	Toxin-coregulated pilus
Tm	Melting temperature
™	Trade Mark
Tris	Tris(hydroxymethyl)aminomethane
TSI	Triple sugar iron
UL	Unlabelled
UNICEF	United Nations Children's Fund
UP	Up converting phosphors
UV	Ultra-violet
w/v	Weight/volume
WHO	World Health Organisation
WHRC	Water and Health Research Centre
^{32}P	Radioactive isotope of phosphorous
µl	Microlitre
µg	Microgram
µm	Micrometre
®	Registered
°C	Degrees Celsius

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CHAPTER 1
STUDY INTRODUCTION AND OBJECTIVES

1.1 GENERAL INTRODUCTION

Escherichia coli, *Salmonella*-, *Shigella*- and *Vibrio* species are responsible for diarrhoeal incidences and outbreaks upon consumption of contaminated food or water. The presence of these pathogens is detected either from the source or faecal matter with microbiological methods and biochemical tests. Despite being the gold standard, culture methods are tedious and time-consuming (Tominaga, 2018). To reduce the time taken for identification and genotyping molecular methods employing nucleic acid extraction and amplification is preferred. Techniques such as the polymerase chain reaction (PCR), real-time PCR and DNA sequencing have demonstrated faster turn-around times and greater sensitivity as a result of millionfold amplification of specific nucleic acid sequence (Li et al., 2018). The sample is processed by extracting and purifying the bacterial DNA, amplification of the target genes with PCR assays and the amplified sequences are visualised on agarose gel electrophoresis.

The electrophoretic pattern identifies the target genes according to migration and separation of the PCR products. Electrophoresis requires the preparation of agarose gels, use of carcinogenic dyes and UV light for visualisation thus limiting its use to laboratories. An alternative option to eliminate the need for large equipment and hazardous materials as well as rapid detection outside of a laboratory is the lateral flow immunoassay (LFIA). An LFIA device is designed for the selective detection and visualisation of amplified target genes by colour change on the test strip (Rohrman et al., 2012; Kamphee et al., 2015; Roth et al., 2018). It requires minimal expertise; is cost effective, rapid and reliable and can be used in both laboratories and in field work.

LFIAs are available for a variety of food and chemical assays; however, no assay is available for the detection of entero-pathogens commonly isolated during outbreaks in South Africa (Posthuma-Trumpie et al., 2009). Entero-pathogens such as *Salmonella*, *Vibrio*, *Shigella* and *E. coli* species have been detected during diarrhoeal outbreaks. To improve the detection and identification of these pathogens, genes can be amplified using polymerase chain reaction and detected using a LFIA.

1.2 OBJECTIVES OF THE STUDY

The following objectives will be used to achieve the aim of the study:

1. Design the lateral flow immunoassay (LFIA) test strips for *Salmonella*, *Shigella*, *Vibrio* and *E. coli* species.
2. Lateral flow immunoassay test strips manufacture and testing with reference bacterial strains
3. Validation and environmental testing of the LFIA test strips

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

A great cause for concern in Southern Africa is the infection and spread of diarrhoeal disease amongst rural communities. The symptoms are generally left unattended in the hope of abating without the need for medical treatment and thus in many cases leads to the loss of life. This is more so likely when the infected individuals are from the high risk category namely the aged, children below the age of five years and the immunocompromised (Gebru et al., 2014). The World Health Organisation (WHO) has defined diarrhoeal disease as the passage of three or more loose/liquid stools per day, besides the normal daily passage and laxative action (WHO, 2008). Despite diarrhoeal disease being non-discriminatory, it has been more prevalent in communities of developing countries. This places a greater disease burden on the socio-economic and health sectors of these communities and has been linked to the limited or no access of clean drinking water, inadequate water supplies for sanitation and personal hygiene (Clasen et al., 2004; Qureshi and Mohyuddin, 2006; Ahs et al., 2010). The primary route of infection with diarrhoeal pathogens has been demonstrated in various studies as the faecal-oral route. Poor sanitation is the primary source of faecal pathogens that are responsible for most diarrhoeal outbreaks. This may be due to unhygienic practices, lack of education, low income settings and other factors (Gebru et al., 2014; Brown et al., 2013). The causative pathogens can range from the viral, bacterial or protozoan groups; however the most common and easily identifiable pathogens are the bacterial type.

Studies conducted by researchers across the globe have shown the prevalence of pathogenic *E. coli*, *Salmonella*-, *Shigella*- and *Vibrio* spp. during diarrhoeal incidences. These pathogens were demonstrated as the commonly occurring enteric pathogens from both diarrhoeal and non-diarrhoeal faecal samples during a study conducted in the Vhembe district of the Limpopo province, South Africa (Mieta, 2009). The recurring isolation and identification of these enteric bacteria when processing faecal and water samples from outbreak affected communities is also indicative of their prevalence in this developing region.

The detection and isolation of these bacterial pathogens are focused on microbiological techniques in most laboratories with the exception of a few specialised laboratories which utilise molecular detection methods. Some of the disadvantages of microbiological analysis is the extensive isolation procedures, long turnaround times and risk of culture contamination when faecal samples are analysed. These factors delay the time taken to provide a confirmed result and thus impacts on the treatment time needed by the infected individuals.

Molecular methods are more favoured as the time needed to obtain confirmed results is shorter than microbiological analysis, however the high cost, specialised equipment and trained individuals are required for the use of assays such as polymerase chain reaction (PCR), DNA sequencing and real-time PCR. Despite the array of techniques available for the detection of bacterial pathogens from different sources the availability of an easy to use, rapid detection assay which can be used in conjunction with conventional PCR is limited. Hence this proof of concept study was conducted to assess the possible development of a multiplex antibody based lateral flow immunoassay for the detection and visualisation of specific antibody-tag labelled PCR amplicons.

2.2 LATERAL FLOW IMMUNOASSAY

The lateral flow immunoassay is a paper-based detection system for the qualitative or semi-quantitative monitoring of analytes wherein samples are placed onto a test strip and the results are displayed by a colour change (Koczula et al., 2016; Wong and Tse, 2009). Lateral flow immunoassays (LFIAs) is an evolving technology that has spread beyond the clinical diagnostics arena into areas such as veterinary, agriculture, food, environmental health and molecular diagnostics in recent times (Wang et al., 2007; Lazcka et al., 2007; Posthuma-Trumpie et al., 2009; Yang et al., 2010; Fill, 2012). A wide variety of point-of-care (POC) immunoassays are currently available in the market segments for monitoring pathogens, drugs, hormones and metabolites in biomedical, veterinary and feed samples within environmental settings (Posthuma-Trumpie et al., 2009; O'Farrell, 2009).

The technical basis of the first LFIA was derived from the latex agglutination assay in 1956 by Plotz and Singer, with the detection of human chorionic gonadotrophin (hCG) also commonly known as the pregnancy strip test being the main application of LFIA technology in the 1970s. Verheijen (2002) describes immunoassays as analytical measurement systems that use antibodies as test reagents. The constant in all these assays is the formation of a complex between the detector reagents, sample and capture reagent (Blažkovă et al., 2009). LFIAs produce accurate, rapid results, easy to use with an extended shelf life hence many assays are available on the market, yet their potential use elsewhere is continuously being investigated and developed.

The principle of lateral flow immunoassays can be explained as the movement of sample from the proximal end of a test strip along the polymeric material whereby it passes and binds to particulate conjugates immobilised at different regions along the test strip (Koczula et al., 2016; O' Farrell, 2009). The configuration of LFIA as shown in Figure 1 demonstrates the design and composition of a typical LFIA. It comprises a sample pad for the application of the sample,

conjugate pad containing the particulate conjugate such as colloidal gold, latex particle, etc., a nitrocellulose membrane to which the biological compound such as antibodies or antigens are immobilized at the test and control line regions and lastly the absorbent pad whose function is to wick excess reagents and prevention of sample backflow (Koczula, 2016; Petryayeva and Algar, 2015).

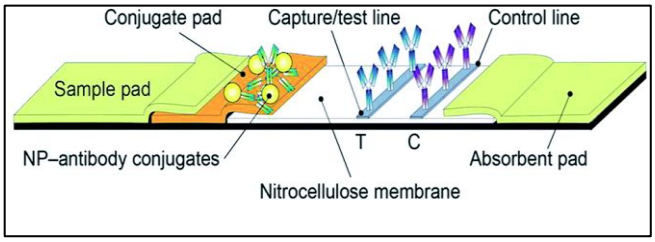


Figure 1 Diagram showing the configuration of lateral flow immunoassay (taken from Petryayeva and Algar, 2015).

LFIAs can be either a competitive (inhibition) or direct (sandwich) format (Fig 2.) (Koczula, 2016; Salieb-Beugelaar and Hunziker, 2014). A competitive format is used when testing for analytes with low molecular weights or single antigenic determinants (Salieb-Beugelaar and Hunziker, 2014; Ngom et al., 2010). Free analytes in a sample compete with the immobilized analytes on the test line to bind with the colloidal gold conjugated antibodies at a defined concentration (Zhang et al., 2009). The result is inversely proportional to the real analyte concentration (Abera, 2010). A positive result is indicated by the absence of a test line; however, a control line should form irrespective of the test line result.

Direct sandwich assay formats are employed to test for larger analytes with multiple antigenic sites (Salieb-Beugelaar and Hunziker, 2014). The target analytes are recognised by antibody conjugate forming analyte-antibody complexes and is bound to the immobilized antibody on the test line, while the excess conjugate is trapped by an antibody on the control line (Zhang et al., 2009). A positive result is determined by the presence of both the test and control lines (O'Farrell, 2009).

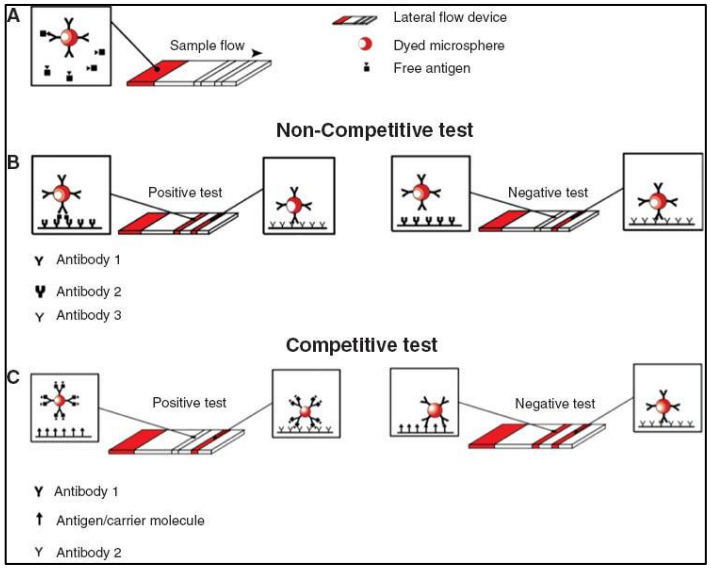


Figure 2 The predominant assay formats used in LFIAs. B) Direct sandwich assay format and C) Competitive/ indirect assay format (Taken from Salieb-Beugelaar and Hunziker, 2014)

Depending on the recognition elements used in an assay it is catergorised accordingly. The common use of antibodies as a detection mechanism for the presence or absence of the compound of interest has been termed lateral flow immunoassays (LFIA) whereas the detection of PCR amplicons using a lateral flow assay is known as the nucleic acid lateral flow immunoassay (NALFIA) (Koczula and Gallotta, 2016; O'Farrell, 2009; Mens, 2008; Horng et al., 2006). Recent technologies have been developed on the basis of LFIAs such as nucleic acid lateral flow immunoassays (NALFIA). NALFIA was designed to detect the presence of amplified double stranded DNA specific to the organism being analysed (Blazkova et al., 2009). The set-up differs from LFIA in that the analyte is an amplified double stranded nucleic acid sequence of a specific organism. The double stranded sequence is amplified using specific primers labelled two different tags (e.g. fluorescein and biotin) (Horng et al., 2006; Posthuma-Trumpie et al., 2009). Antibodies raised against the tag (fluorescein) are sprayed onto the test line (Chun 2009; Noguera et al., 2011). Recognition will occur when the analyte is bound to the anti-tag on the test line and the visualisation is achieved by the biotin labelled analyte binding to gold nanoparticles labelled with avidin hence producing a reddish colour (Assadollahi et al., 2009; Blazkova et al., 2009). The dye tags are chemical structures which attach to the specific primers via chemical linkers such as aminoethylcarbamoyl. This assay is a fast and simple one step assay, thus does not require any washing steps as with reverse hybridization assays (Mens, 2008; Posthuma-Trumpie et al., 2009)

2.3 COMPONENTS OF A LATERAL FLOW IMMUNOASSAY

The components required for the design and construction of a LFIA are antibodies, the sample pad, conjugate pad, detection pad, wick/absorbent pad and backing material. The following four zones are required to obtain a functional LFIA, ❶ sample application pad to carry the sample via the ❷ conjugate pad containing the labelled analyte/ recognition element to the ❸ detection zone with the embedded test and control lines for visible detection and the ❹ absorbent pad removes the excess unbound sample from the LFIA strip.

Sample Pad

The sample pad functions to distribute a sample onto the conjugate pad evenly thereby preventing the flooding of the strip. This is generally achieved by pre-treatment of the sample pad material with buffer salts, surfactants, etc. which controls the flow rate of samples across the surface (Koczula and Gallotta, 2016; Sajid et al., 2015). Materials selected to function as the sample pad are dependent of the type of samples to be assayed and should have good tensile strength when wet amongst other factors (O'Farrell, 2009). Commonly used materials for LFIAs range from rayon, cellulose or glass fibre depending on the application of the assay.

Conjugate Pad

The role of a conjugate pad is to accept the detector reagent and retain it for the shelf life of the test strip. Upon use of the tests strip the detection reagent is to be released, solubilised and the complex is transferred onto the detection zone during the flow of the sample across the assay (O'Farrell, 2009; Sajid et al., 2015). To achieve an optimal release of the detector reagent from the pad, the pad may be impregnated with surfactants, proteins or polymer solutions and dried prior to application of the detector reagent (Koczula and Gallotta, 2016; O'Farrell, 2009). The detector reagent is applied to the conjugate pad by immersion of the pad into the reagent suspension or dispensing the reagent onto the pad. The materials are generally selected based on the properties needed for efficient reagent release during an assay (Table 1).

Labels/ detection Conjugate

The visualisation of analytes is achieved by the use of labels. Initially enzymes were used for labelling in enzyme immunoassays; however, they were soon replaced by particulate labels (Posthuma-Trumpie et al., 2009). Particulate labels are made of nanoparticles which range from 15-800 nm and could be coloured or fluorescent.

Table 1 Properties of conjugate pad materials

Material	Description	Strength	Weakness
Glass fibers	• 100-500 µm thick • May contain binders to hold fibers together	• Good hold-up volumes • Low non-specific binding	• Poor tensile properties • Difficult to slit and web handle
Cellulose fibers	• 300-1000 µm thick • Compact fibers of consistent density	• Very low non-specific binding • Uniform	• High hold-up volumes (>50µL/cm^2) • Can be very weak when wet
Surface-modified polyester	• 100-300 µm thick • Hydrophilic polyester filters	• Low non-specific binding • Excellent tensile strength and web handling	• Low and variable hold-up volumes (<15µL/cm^2)

The use of nanoparticles has given LFIAs a great boost in terms of enhancing its sensitivity and multiplexing ability (Kaittanis et al., 2010). Labels commonly employed in assays such as colloidal gold, carbon, latex, magnetic nanoparticles, liposomes and more recently fluorescent nanoparticles, quantum dots and up-converting phosphorous technology are described in Table 2 (Assadollahi et al., 2009; Chun, 2009; Kaittanis et al., 2010; Ngom et al., 2010).

Table 2 Types of labels available/used in lateral flow immunoassays

Label	Description	Reference
Colloidal gold	• Widely used and commercially available • Intense colour change • Stable in liquid and dried forms	• Bahadır and Sezgintürk, 2016 • Koczula and Gallotta, 2016 • Sajid et al., 2015 • Virekunnas, 2012 • Fournier-Wirth and Coste, 2010
Latex particles	• Versatile and easy to purchase • Tagged with various detector reagents	• Koczula and Gallotta, 2016 • Chun, 2009
Carbon particles	• Economic, high colour contrast • Good stability	• Sajid et al., 2015 • Chun, 2009
Magnetic nanoparticles	• Used as coloured labels by producing colour at test line	• Sajid et al., 2015 • Mohamad Nor et al., 2012

Label	Description	Reference
	• Magnetic signals more stable than optical signals • Enhance sensitivity 10-1000 fold	
Quantum dots	• Uniform in size • Resistant to photobleaching • Easily amenable to multiplexing • Wide excitation and narrow emission spectrum • High background noise	• Fournier-Wirth and Coste, 2010 • Kaittanis et al., 2010

Membrane/ Detection Region

The detection region is crucial and vital element of the as assay strip. This area functions to bind molecules at the test and control areas and maintain their stability for short- or long-term storage (Table 3). During the assay this region enables the sample-conjugate to bind to the corresponding immobilised proteins/antibodies at the correct test or control line which is displayed by the presence of a colour line (Sajid et al., 2015; O'Farrell, 2009).

Nitrocellulose membranes are the common choice for many assays as it meets the required parameters needed to design a functional LFIA (Sajid et al., 2015). However, the choice of membrane is greatly dependent on the capillary flow rate of the lateral flow assay. This is determined by the time required for a liquid to travel across and completely fill the strip of membrane, defined as the capillary flow time (Koczula and Gallotta, 2016).

Table 3 Binding properties of membranes available for immunoassays

Type of Membrane	Mechanism of binding capture reagents
Nitrocellulose	Electrostatic
Polyether sulphone	Hydrophobic
Nylon	Electrostatic
Polyvinylidene fluoride (PVDF)	Hydrophobic

Test and Control Line Reagents

Antibodies or immunoglobulins recognize specific substances efficiently with antigen binding sites and hence are utilized in recognition systems. Immunoassays function by immobilizing high affinity capture ligands onto a membrane. These ligands are either antibodies or proteins

which capture proteins or autoantibodies of interest within the sample (Ellington et al., 2010). Hence antibodies are a critical component in the development of LFIAs. Antibodies are classified according to their synthesis and selective recognition properties (Lazcka et al., 2007). Monoclonal antibodies are produced from one hybridoma cell thus it will always recognize the same epitope of the antigen. Polyclonal antibodies are synthesised from the whole serum thus they will recognize different epitopes of the antigen. Recombinant antibodies are created by introducing the DNA coding for a required fraction of the antibody into bacteria. The fractions are produced and extracted from the bacteria. The antibodies selected for a particular assay should be purified properly and screened for the highest affinity and specificity (Volkov et al., 2009). If any of the components are suboptimal the assay will have a limited performance capacity.

Immobilisation of the antibodies at the test and control line sections influences the quality of the test. Three factors need to be optimised for results to be readable with the naked eye; firstly, the concentration of protein/antibody bound needs to be sufficient. Secondly the method used to immobilise the antibodies onto the membrane needs to be optimised and lastly the binding of the capture antibodies to the membrane needs to be strong. The common immobilisation methods utilised are ❶adsorption on gold, ❷avidin-biotin system and ❸self-assembled monolayers (SAMs). Adsorption on gold is the random attachment of antibodies onto the substrate. This method is the quickest but least reliable as the correct orientation of binding sites are uncontrolled. According to Tombelli and Mascini (2000) the adsorption is non-specific and biosensor performance is poor. The avidin-botin system is a simple yet effective immobilisation method onto an avidin coated surface. This can be attributed to the high affinity constant between biotin and avidin (Tombelli and Mascini, 2000). It has been successfully used however there is a high cost for the reagents used. Self-assembled monolayers were first reported by Bain and co-workers in 1989. The SAMs are obtained by immersing a gold plate into a solution containing a suitable surfactant in a high purity solvent. After the monolayer formation the bio-molecule is attached to the other end of the thiol molecule. This technique is very robust therefore it can be found in a wide variety of applications. In nucleic acid assays (NALFIA) an antibody tag-label interaction and avidin-biotin system is used (Posthuma-Trumpie et al., 2009).

Absorbent pad/ Wick

The absorbent pad is required to wick the excess sample through the test strip and hold it for the duration of the assay thereby lowering the background and enhancing assay sensitivity (Koczula and Gallotta, 2016; Sajid et al., 2015). The wicking material is generally high-density cellulose for efficient absorption of the liquid.

Adhesive Card/Backing Card

All the above-mentioned components of the LFIA are bound to backing material for easy handling and rigidity of the test strip (Sajid et al., 2015). Typical backing materials used are polystyrene, vinyl or polyester coated with medium to high tack adhesive. The backing is required to enable lamination of the multiple materials into one unit.

CHAPTER 3
MATERIALS AND METHODS

3.1 Bacterial strains

The bacterial strains used for the experimental work (Table 2.1) were obtained from the National Health Laboratory Services (NHLS), American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC). All the bacterial strains were stored at -70°C in Microbank™ cryo-vials (Pro-Lab Diagnostics, USA) prior to use.

Table 4 Bacterial strains used for experimental work

Micro-organisms	Source	Micro-organisms	Source
Escherichia coli (Commensal)	NHLS	*Shigella dysenteriae* type 1	NHLS
Enterohaemorrhagic *E. coli*	NHLS	*Shigella flexneri*	NHLS
Enterotoxigenic *E. coli*	NHLS	*Shigella boydii* serotype B	NHLS
Enteroaggregative *E. coli*	NHLS	*Shigella sonnei*	NHLS
Enteroinvasive *E. coli*	NHLS	*Salmonella typhimurium*	NHLS
Enteropathogenic *E. coli*	NHLS	*Salmonella typhimurium*	NHLS
Vibrio cholerae non-O1	NHLS	*Salmonella enteritidis*	NHLS
Vibrio cholerae O139	NHLS	*Salmonella typhi*	NHLS
Vibrio fluvialis	NCTC	*Salmonella typhi*	NHLS
Vibrio cholerae O1	NCTC	*Salmonella paratyphi*	NHLS
Vibrio parahaemolyticus	NHLS	*Salmonella paratyphi* C	NHLS
Klebsiella pneumonia	NHLS	*Salmonella gallanarum*	NHLS
Morganella morganni	NHLS	*Enterococcus faecium*	NHLS
		Enterococcus faecalis	NHLS

3.2 DNA Extraction Methods

DNA was isolated from bacterial strains and various sample types according to the modified GuSCN DNA extraction protocol reported by Mieta and co-workers (2010). The eluted DNA was kept at -20°C until use.

3.3 Polymerase Chain Reaction (PCR)

A modified genus specific multiplex Polymerase chain reaction (gm-PCR) assay described by Mieta and co-workers (2010) was used as the basis for the amplification of the species specific genes for enteroinvasive *E. coli* (EIEC), *Vibrio*-, *Salmonella*- and *Shigella* species. The *sodB* gene was targeted for *Vibrio cholerae*, *IpaB* gene for *Salmonella* spp., the *IpaH* gene for *Shigella* spp. and the *Ial* gene for EIEC (Mieta et al., 2010). The primers synthesised for the amplification of the selected genes were labelled with specific antibody tags to enable recognition and binding at the correct positions on the LFIA test strip (Table 5). DNA isolated from the bacterial suspensions was used as DNA templates during the development and validation of LFIA. The DNA templates were quantified prior to amplification using the Qubit™ Quantification Platform Fluorometer (Invitrogen; USA).

Table 5 Primers used for the genus specific PCR assay

Primer	Sequence (5'→ 3')	Product length (bp)	Reference
Entero-invasive *E. coli* (EIEC)/ *Shigella* spp. primers			
IpaH-F *IpaH*-R	**Biotin**-CCT TGA CCG CCT TTC CGA TA **Texas red** X-CAG CCA CCC TCT GAG GTA CT	606	Kong et al., 2002
***Salmonella* spp. primers**			
IpaB-F *IpaB*-R	**Biotin**-GGA CTT TTT AAA AGC GGC GG **Alexa fluor**-GCC TCT CCC AGA GCC GTC TGG	314	Kong et al., 2002
***Vibrio* spp. primers**			
SodB-F *SodB*-R	**Biotin**-AAG ACC TCA ACT GGC GGT A **Bodipy FL**-GAA GTG TTA GTG ATC GCC AGA GT	248	Tarr et al., 2007

For each PCR, the reaction mixture consisted of 1X Qiagen multiplex PCR master mix (Qiagen, Germany). To this 100 pmol of each labelled primer; 2 µℓ of the DNA template and PCR grade water were added to make up the final volume. A volume of 2 µℓ DNA consisting of V*. cholerae* O1, S*. dysenteriae* or *S. typhimurium* DNA mixture was included as a PCR positive control template and the negative control was included without template DNA. This was included to test for the presence of possible background appearance or false positives. The reaction mixture was subjected to the following amplification cycle conditions; a single initial denaturation cycle at 95°C for 15 minutes, followed by 35 cycles consisting of ❶ denaturation at 94°C for 45 seconds ❷ annealing at 57°C for 45 seconds and ❸ elongation at 72°C for 1 minute and a final elongation step at 72°C for 5 minutes. All PCR reactions were performed in a Biorad Mycycler™ Thermal cycler. After completion of PCR amplification the amplified DNA was used for LFIA detection and agarose gel electrophoresis ([2.5% (w/v)] with ethidium

bromide (0.5 µg/ml) in TAE buffer [40 mM Tris acetate; 2 mM EDTA, pH 8.3]) for comparative results.

3.4 Lateral Flow Immunoassay (LFIA) Test Strip Development

A sandwich format lateral flow immunoassay was designed and developed as an alternative to the visualisation of amplified PCR products using the gel electrophoresis system. The selected bacterial pathogens to be screened were *Salmonella*-, *Shigella*-, EIEC and *Vibrio* species. The capture reagents chosen were based on the literature of previously successfully developed LFIAs (Horng et al., 2006; Soo et al., 2006; Soo et al., 2009; Noguera et al., 2011). Colloidal gold particles conjugated with streptavidin were used for the detection conjugate as the assay utilised the avidin-biotin and antibody tag-label recognition systems (Figure 3).

Design and optimisation of the LFIA comprised of component material selection and preparation, optimal conjugate label volume for sufficient colour intensity, antibody volume and concentration to be blotted onto nitrocellulose membranes, sample volume required to flow across test strip and lastly the assembly of the test strips.

Preparation of assay components

Glass fibre sheets (Millipore, MA) were selected as the material for sample and conjugate pads (Horng et al., 2006; Soo et al., 2006). The GF sheets were cut into 10 mm x 6 mm pieces for the sample pads and kept covered until assembly, whereas the conjugate pads were cut into 5 mm x 6 mm pieces and blotted with 10ul of 40 nm colloidal gold coated streptavidin (BioAssay Works, USA).

Cellulose fibre pads were used as absorbent pads for effective wicking of excess sample. 10 mm x 6 mm pieces were cut from the cellulose fibre pads and kept covered until assembly. Vivid 170 nitrocellulose (NC) membranes (Pall Life Sciences, USA) were cut into 40 mm x 0.6 mm strips. The membrane was pre-wetted with 6X sodium, sodium citrate solution (20X SSC; 3M NaCl, 0.3M trisodium citrate) for 10 minutes on the IKA® KS 260 rotational shaker (IKA, Germany) to ensure complete hydration of the membrane. The membranes were thereafter placed onto moistened filter paper on the Bio-Dot® SF Microfiltration apparatus ready for application of test and control reagents.

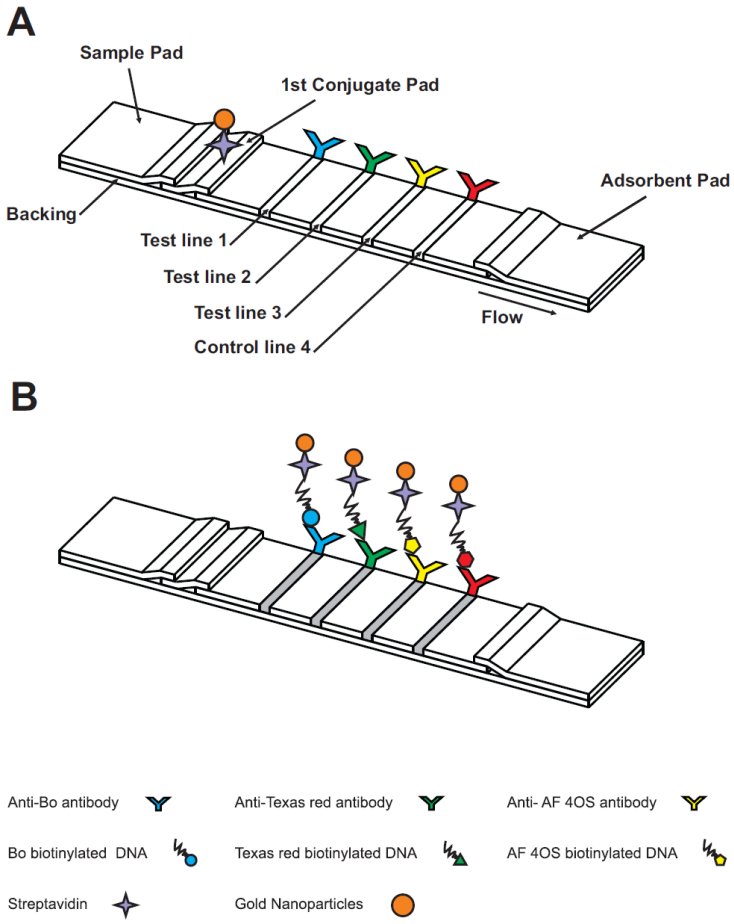


Figure 3 Schematic representation of the lateral flow immunoassay test with colloidal gold nanoparticles before (A) and after (B) use.

Test and Control Line Optimisation and Application

Prior to application of the test and control reagents the nitrocellulose membranes were rehydrated with 50 µℓ 1X TBS buffer (tris-buffered saline, pH7.5; 20 mM Tris, 500 mM NaCl) for 30 minutes. All test and control line antibodies were purchased commercially (Invitrogen, USA and Vector Laboratories, USA). The Fluorescein (F), Texas Red (TxR), Alexa-Fluor 405 (AF) and Bodipy® FL (BO) antibodies were supplied in a solution of PBS, pH 7.2 containing 5 mM sodium azide. The biotinylated anti-mouse IgG antibody (Vector Laboratories, USA) was shipped in a lyophilised form and was reconstituted with 1 mℓ PCR grade water upon use. All antibodies were stored at 4°C.

Test strips were prepared with varying dilutions of each antibody and the dilution that produced optimal colour intensity was selected (Table 6). The volume of each antibody to be blotted was

also determined accordingly. The working volume of PCR product to be added to flowing buffer was kept constant at 10 µℓ throughout the study.

Table 6 Antibody dilution range selected for colour intensity optimisation

ANTIBODY	[CONC]	DILUTIONS				
Fluorescein(F)	0.5 mg/ml	1:1000	1:500	1:100	1:50	1:10
Texas Red (TxR)	1 mg/ml	1:1000	1:500	1:100	1:50	1:10
Alexa-Fluor 405 (AF)	3 mg/ml	1:1000	1:500	1:100	1:50	1:10
Bodipy® FL (BO)	3 mg/ml	1:1000	1:500	1:100	1:50	1:10

The test and control lines were blotted onto the nitrocellulose membrane using the Bio-Dot® SF Microfiltration apparatus (Bio-Rad, USA) attached to a Vacuubrand® vacuum (Figure 4) at a distance of 8 mm between each line. The antibody stock solutions were diluted 10 and 50 times in cold PBS; pH 7.4 and a final volume of 50ul blotted onto the nitrocellulose membrane. Once all antibodies were applied the membrane was blocked with a 1% (w/v) casein enzymatic hydrolysate solution (Sigma Aldrich, St. Louis, MO) to prevent non-specific protein binding onto the membrane.

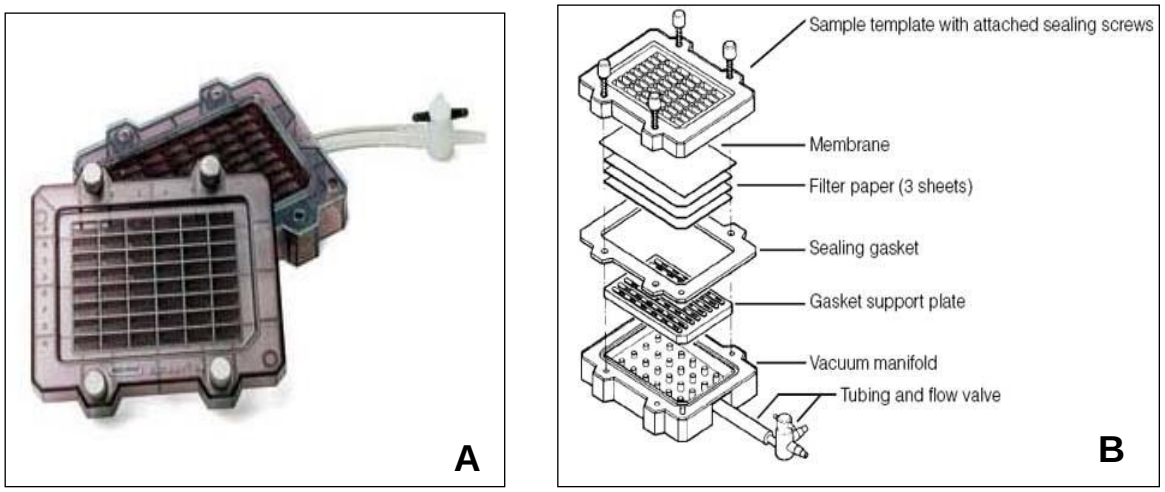


Figure 4 A) Image of Bio-Dot® SF Microfiltration apparatus and B) components and assembly guide for the apparatus. (Images adopted from Bio-Rad instruction manual)

Lateral Flow Immunoassay (LFIA) Assembly

The LFIA strip was made functional by assembling the nitrocellulose membrane, conjugate, sample and absorbent pads onto a support card (purchased from local store). The

nitrocellulose membrane was attached to the support card with adhesive at a distance of 15 mm from the top end of the support card. The conjugate (5 mm x 6 mm) and absorbent pads (10 mm x 6 mm) were placed on either end (top and bottom end) of the nitrocellulose with an overlap of 2 mm onto the nitrocellulose membrane. Lastly the sample pad (10 mm x 6 mm) was attached overlapping the conjugate pad (Figure 5). The support card was cut to size and the strips were used as required.

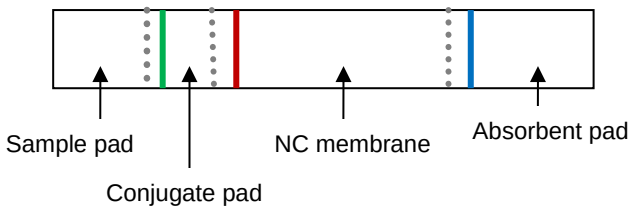


Figure 5 Schematic diagram of LFIA strip assembly (top view); Overlap

Ten microlitres of the labelled amplicon product was mixed with 90 µℓ flowing buffer (PBS, pH7.4) and applied onto the sample pad. The sample migrated onto the conjugate pad, interacted with the colloidal gold particles and the complex flowed onto the nitrocellulose membrane. After 15 minutes at ambient temperature the test strip was read for the presence or absence of the control and test lines. Antibody tag-label interactions at the test line resulted in a visible red line being produced. No red line was present in a negative sample. The control line was formed by trapping the excess gold conjugates to the immobilised biotinylated antibody. A definite red line had to appear at the control line irrespective of the test line outcome. If no control line was visible the test strip was invalid and the sample retested. A negative sample control comprising of PCR grade water was added to the flowing buffer and applied to the test strip.

3.5 Validation of the Lateral Flow Immunoassay Test Strip

The test strips were validated in terms of reliability, specificity, sensitivity, precision and accuracy.

Reliability of the assay was evaluated by the reproducibility, precision and accuracy of the test strip. Precision is defined as “the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions” (Garofolo, 2004). Thus to determine the precision and accuracy of the LFIA a set of three independent amplified PCR products, positive for *Salmonella*-, *Shigella*- and *Vibrio* spp. general genes were analysed on the same day on the LFIA strips of the same batch. The results obtained were validated against the gel electrophoresis results for the same samples.

Reproducibility studies consisted of three independent repeats of triplicate amplified PCR products for each organism tested on LFIA strips produced in three different batches. The results of each day were compared and validated. The reproducibility of a test is generally determined by measuring the precision between laboratories. For validation of the LFIA reproducibility for this study was defined as the precision of the method under the same operating conditions over a short period of time (Garofolo, 2004).

The specificity/selectivity of a method is defined as the ability of the method to measure and differentiate between the analyte and closely related substances (Garofolo, 2004; Eurachem, 1998). In order to correctly validate the selectivity of the LFIA the cross reactivity/specificity of the test strip was studied by testing the target bacteria and other bacteria as listed in Table 4. The bacteria were grown overnight in nutrient broth at 37˚C with agitation at 200rpm. DNA was extracted, amplified with the labelled gm-PCR and the products visualised on gel electrophoresis and LFIA.

According to the International Union of Pure and Applied Chemistry (IUPAC) sensitivity can be defined as the measure of response caused by a certain amount of analyte (IUPAC, 1999). In order to determine the sensitivity of the test strips amplified PCR products for each organism were quantified using the broad range DNA quantification kit (Qubit fluorometer, Invitrogen USA). The samples were serially diluted and the diluted samples were analysed on the test strips. Each dilution was analysed in triplicate for all three amplified genes. The limit of detection (LOD) was determined as the lowest concentration that produced a visible positive test line.

3.6 Field Application

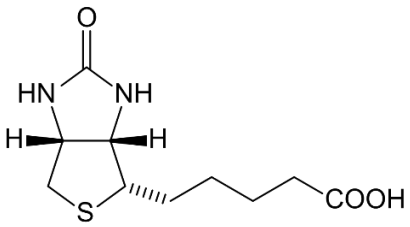
Hundred and thirty-eight DNA samples originating from wastewater (n=62), animal feed (n=7), dishcloths (n=11) and fly samples (n=58) were analysed on LFIA and agarose gel electrophoresis. The samples were previously analysed and hence confirmed results were available. A negative control was included for every batch of test strip used. The results from both methods were compared and interpreted accordingly.

CHAPTER 4
RESULTS AND DISCUSSION

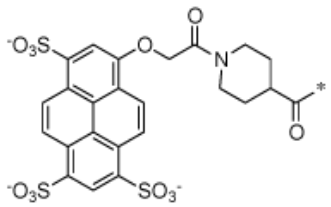
4.1 POLYMERASE CHAIN REACTION

To determine the functionality of the LFIA, the PCR assay incorporated labelled primers for the amplification of the genes. As shown in Table 5 the forward primers labelled with biotin at the 5' position and the corresponding reverse primers were labelled at the 5' position using dye-tags. The chemical structures of dyes conjugated to primers vary which could modify the product size (Figure 6). Therefore, the genes (*sodB*, *IpaB* and *IpaH*) were amplified independently with labelled and unlabelled primers according to the gm-PCR amplification conditions. The *sodB*, *IpaB* and *IpaH* genes were successfully amplified with using both labelled and unlabelled primers and confirmed on agarose gel electrophoresis. The product lengths for all three genes remained the same for both primer types thus confirming that the dye tags did not influence the final product size (Figure 7).

Biotin

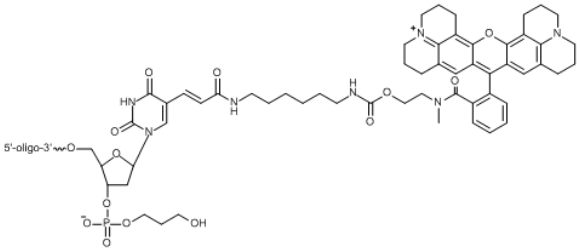


Alexa Fluor 405

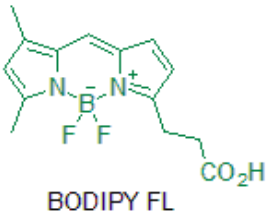


Alexa Fluor® 405

Texas Red



BOdipy® FL



BODIPY FL

Figure 6 Chemical structures of dye tags used for Nucleic Acid Lateral Flow Immunoassays (NALFIAs) (Biotin: www.chm.bris.ac.uk; Alexa Fluor 405 and BOdipy®FL: https://www.atdbio.com; Texas Red: https://www.biosearchtech.com)

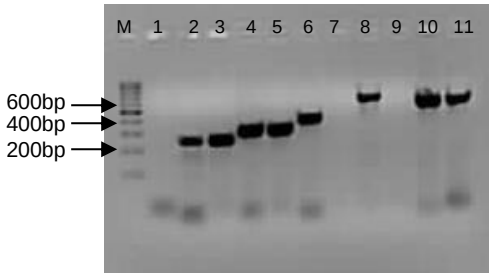


Figure 7 Agarose gel showing gm-PCR single genes amplified with unlabelled (UL) and labelled (L) primers. Lane M indicates the 100bp Fermentas O' GeneRuler DNA ladder run. Lane 1- negative control; Lane 2- 248bp *sodB* gene (UL); Lane 3- *sodB* gene (L); Lane 4- 314 bp *IpaB* gene (UL); Lane 5- *IpaB* gene (L); Lane 6- 392bp *Mdh* gene(UL); Lane 7- *Mdh* gene (L); Lane 8- 630bp *Ial* gene (UL); Lane 9- *Ial* gene (L); Lane 10- 606bp *IpaH* gene (UL); Lane 11- *IpaH* gene (L)

The presence of the single genes on PCR warranted the labelled primers of all three genes be combined into a single assay for the multiplexed PCR assay. Figure 8 demonstrates the amplification and detection of all three genes using agarose gel electrophoresis. Two bands (*IpaH* and *SodB*) instead of three were observed in lane 2 when a lower volume of labelled primers used. This may have been due to either ❶ non-optimal primer concentration or ❷ the primers may have been insufficient for amplification or ❸ they may have degraded during amplification. From this observation it was recommended that a final 1µℓ primer volume be used for all future amplifications.

Upon successful PCR optimisation it was concluded that the gm-PCR using labelled primers for the amplification of *sodB*, *IpaB* and *IpaH* could detect the presence of *V. cholerae*, EIEC, *Salmonella*- and *Shigella* species.

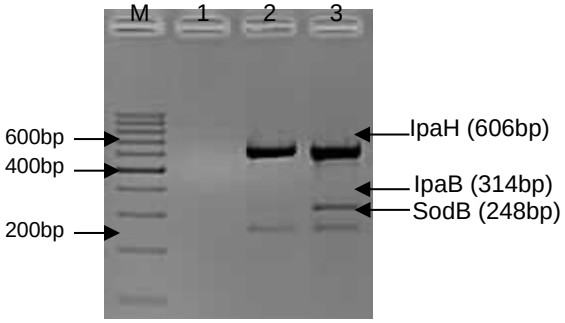


Figure 8 Agarose gel showing gm-PCR multiplex genes amplified labelled (L) primers. Lane M indicates the 100bp Fermentas O' GeneRuler DNA ladder run. Lane 1- negative control; Lane 2- mPCR tube 1 ; Lane 3- mPCR tube 2.

4.2 LATERAL FLOW IMMUNOASSAY (LFIA)

The lateral flow immunoassay was designed and validated for the detection of EIEC, *Vibrio*-, *Salmonella*- and *Shigella* spp. Manufacture of the LFIA test strip entailed membrane selection, concentration of colloidal gold nanoparticles and antibodies to be blotted and assembly of the test strip. Once a functional LFIA strip was created its ability to detect the selected PCR products was assessed by subjecting it to a validation study.

The test strip comprised of a sample pad, conjugate pad containing the colloidal gold nanoparticles, nitrocellulose membrane (NC) with the anti-dye antibodies blotted at the respective test and control lines and lastly the absorbant pad to wick excess sample from the test strip. The test strip detects the presence of amplified PCR products using primers labelled with two different tags. The biotin tag located at the 5' end of the amplified product forms a complex with the streptavidin colloidal gold which is responsible for the visualisation of a red colour at the test line and the dye tag attaches to the anti-dye antibody immobilised on the nitrocellulose membrane for capture of the amplified PCR product. Test and control lines were blotted in the following order on the test strip (left to right); anti- BOdipy®FL (*V. cholerae*); anti-Alexa Fluor 405 (*Salmonella* spp.); anti-Texas red (EIEC/*Shigella* spp.) and anti-mouse IgG (control).

4.2.1 Membrane Selection

Membranes were selected on the basis of optimal performance and their ability to produce reliable results without inteferences during the assay. All materials used were selected based on reviews and outcomes of studies conducted by manufacturers/researchers in the development of lateral flow immunoassay tests. Factors such as availability of materials, local suppliers and pricing were also included during the evaluation process. All membrane types purchased and optimised for this assay are shown in Table 7.

Table 7 Description of membranes selected for the Lateral Flow Immunoassay and suppliers

MATERIAL TYPE	DESCRIPTION	SUPPLIER
Glass fibre(20 cm x 30 cm)	Material used for sample pad and conjugate pad	Millipore Corporation, Bedford MA
Cellulose Fibre(20 cm x30 cm)	Thicker material used for absorbant pad	Millipore Corporation, Bedford MA
Vivid 170 Nitrocellulose Membrane (25 mm x 50 m)	Membrane used for binding of antibodies at the test region	Pall Life Sciences, USA

4.2.2 Labels

Gold nanoparticles are utilised in many assays as they are stable under different conditions, easily detectable, able to produce reproducible results and are highly specific amongst other factors (Koczula et al., 2016; Karakus and Salih, 2013). They have been found to possess unique optical properties making them suitable for labelling and visualisation in lateral flow immunoassays (Koloslova et al., 2007; Guo et al., 2009; Posthuma-Trumpie et al., 2009; Ngom et al., 2010).

Conjugation of gold nanoparticles to antibodies or labels have been achieved by electrostatic coupling (Ijeh, 2011). For this immunoassay the colloidal gold nanoparticles were conjugated with streptavidin for the detection of biotin labelled amplified products (Horng et al., 2006; Soo et al., 2006; Soo et al., 2009).

The streptavidin conjugated gold (SCG) nanoparticles were purchased commercially and was used in accordance with the manufacturers indications of 10 µℓ volume of a 15OD (~533 nm) concentration per assay. The presence of a red coloured line at the test position of the strip is indicative of presence of target analytes in the sample, hence the line intensity was proportional to the concentration of target analyte bound to the SCG complex. A negative sample will display a line at the control line on the test strip as the SCG nanoparticles binds only to the biotin labelled control antibody.

The production of a red line at the test region of the strip is dependent on the binding efficiency and concentration of both the gold nanoparticles and the anti-dye antibody tags. The required concentration and volume of anti-dye antibody to be blotted was determined by applying increasing volumes (10, 20, 50 and 100 µℓ) of each antibody at the stock concentrations to the nitrocellulose membrane (Table 8). According to the manufacturer the dilutions to be used

should be determined empirically thus the best line intensities were observed with serially diluted stock concentrations.

Anti- BOdipy®FL (anti-BO) recognised the BOdipy®FL dye attached to the 3' end of the *sodB* PCR amplified gene for *V. cholerae* detection. Anti-Alexa Fluor 405/ cascade blue (anti-AF) is the anti-tag to the dye Alexa Flour 405 which was attached to the 3' end of the *IpaB* amplified gene for the detection of *Salmonella* spp. Anti-Texas Red (anti-TxR) recognised and bound to the Texas Red dye located at the 3' end of the amplified *IpaH* gene used for the detection of EIEC/*Shigella* spp. Biotinylated anti-mouse IgG was used as the control for the lateral flow immunoassay.

The test line intensities for each antibody differed in terms of the concentration and volumes coated onto the test strip. A 50 µℓ volume of anti-BO, anti-AF, anti-TxR and anti-mouse IgG (diluted in PBS) applied onto the test strip produced good line intensities (Table 3.7). The stock antibody concentration for TxR was much lower than the anti- BO and anti-AF antibody concentrations; hence the optimal anti-TxR test line intensity was achieved with the application of a 100 µℓ antibody instead (Table 8).

Table 8 Test line intensity optimisation for a) anti-BOdipy®FL [*sodB*] at a concentration of 3 mg/ml; b) anti-Alexa fluor 405 [*IpaB*] at a concentration of 3 mg/ml; c) anti-Texas red [*IpaH*] at a concentration of 1 mg/ml.

a)

3 mg/ml	***1:1000***	***1:500***	***1:100***	***1:50***	***1:10***
10 µℓ	× 	× 	× 	× 	✓ 
20 µℓ	×	×	×	±	±
50 µℓ	×	×	✓	✓	✓
100 µℓ	×	✓	✓	✓	✓

b)

3 mg/ml	***1:1000***	***1:500***	***1:100***	***1:50***	***1:10***
10 µℓ	× 	× 	± 	✓ 	✓ 
20 µℓ	×	±	✓	✓	✓
50 µℓ	×	✓	✓	✓	✓
100 µℓ	✓	✓	✓	✓	✓

c)

1 mg/ml	***1:1000***	***1:500***	***1:100***	***1:50***	***1:10***
10 µℓ	× 	× 	× 	× 	× 
20 µℓ	×	×	×	×	×
50 µℓ	×	×	×	×	×
100 µℓ	×	×	×	×	✓

✓: Band detected

×: No band detected

±: Feint band (not visible in picture)

From the data in Table 8 the selected optimal test and control line intensities for the manufactured test strips were achieved by the application of the following diluted antibody concentrations; 0.15 mg/ml anti-BO (*sodB*), 0.15 mg/ml anti-AF (*IpaB*), 0.05 mg/ml anti-TxR (*IpaH*) and 0.005 mg/ml biotinylated anti-mouse IgG on the nitrocellulose membranes (Table 9; Figure 9).

Although the above-mentioned volumes were used for the lateral flow immunoassay test strip development, factors such as increasing the concentration of all antibodies to 3 mg/ml, applying larger volumes or widening the antibody line on the membrane may improve the intensity of the line colour (Bruning et al., 1999). Due to the limited time available for this study (unforeseen delays in delivery of assay materials) these factors were not included in this study. Thus it is recommended for follow-up studies and improvement of the lateral flow immunoassay test strips that these factors be explored and investigated.

Table 9 Antibody application volume optimisation for anti-BO, anti-AF and anti-TxR

Antibody volume	***Anti-BO(1:10)***	***Anti- AF(1:50)***	***Anti- TxR(1:10)***
20 µℓ 30 µℓ 50 µℓ 100 µℓ			

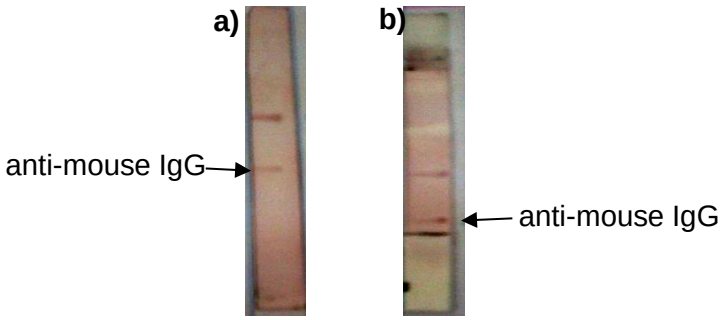


Figure 9 Lateral flow immunoassay test strip showing a) NC membrane blotted with 50 ul of 5 µg/ml anti-mouse IgG and b) location of control line on assembled test strip

4.2.3 Lateral Flow Immunoassay Assembly

To obtain a functional test strip many variables within the components of the test strip need to optimised for harmonisation upon assembly. Factors that greatly influence the final product are the size of test strip, materials used, detection and capture reagents and the sample volume used. The size of a test strip in terms of width and length influences the results obtained from the assay. Basic physics has shown that movement, in this case, of the sample along a larger surface area requires more time and energy. As such the sample volume needed to traverse a greater surface area of the test strip increases. This would not be favourable for this kit because of the small sample volumes of amplified PCR products. Therefore various dimensions as shown in Table 10 were tested to achieve the final dimensions of the lateral flow immunoassay strip.

Table 10 LFIA test strip dimension optimisation showing initial dimensions and final test strip dimension

Components	***Initial dimensions***		***Final dimensions***	
Sample Pad	2 cm x 2.5 cm		1 cm x 0.6 cm	
Conjugate pad	1 cm x 2.5 cm		0.5 cm x 0.6 cm	
NC membrane	5.5 cm x 2.5 cm		3.5 cm x 0.6 cm	
Absorbent pad	2 cm x 2.5 cm		1.5 cm x 0.6 cm	
Assembled strip	**10.5 cm x 2.5 cm**		**6.5 cm x 0.6 cm**	

Initial dimensions tested were reduced as the findings suggested that the sample volume, colloidal gold nanoparticles and antibody volumes were insufficient for the dimensions chosen (Table 10). Upon decreasing the surface area from *10.5 cm x 2.5 cm* to *6.5 cm x 0.6 cm* the

results improved and the cost of the required reagents were minimised. Reducing the dimensions of the test strip showed that the selected volumes were sufficient to provide a defined red colour at both the test and control lines. A mixture of 10 µℓ amplified PCR product and 110 µℓ flowing buffer (PBS, pH 7.4) was applied onto the test strips. The final volume of 120 µℓ was selected as the optimal volume required for efficient analysis. Test strip preparation time for antibody application, drying of conjugate pad and sample volume were reduced in relation to the adjusted strip size.

The duration for complete preparation, assembly and use of the lateral flow immunoassay test strip was approximately 4 hours. However it should be highlighted that the preparation of all materials (cutting, applying test and control lines, blotting conjugate material and assembly) were all conducted manually which therefore resulted in an increased preparation time. For the implementation and field use the automation of these processes will be greatly reduce the time factor. As a result the overall time required to perform this test on available test strips will be 15-30 minutes which is in the similar range of many lateral flow immunoassays designed (Arao et al., 1999; Guo et al., 2000; Aldus et al., 2003; Biaginni et al., 2006; Anfossi et al., 2010).

Test strips were assembled onto a cardboard backing and kept in position with non-toxic general use adhesive. Test strips were not housed in cassettes as the commercially available cassettes viewing panes were not suited for the detection of multiple analytes on the multiplex lateral flow immunoassay strip. Possible future development of the lateral flow immunoassay into a commercially available product will warrant detailed R&D studies during which these factors will be thoroughly investigated.

4.2.4 Visualisation of PCR Amplified Products on Lateral Flow Immunoassay

Individual and multiplexed amplified PCR products were visualised using agarose gel electrophoresis and lateral flow immunoassay and the results compared. Gene amplified PCR products (10 µℓ) were mixed with 10 µℓ SCG and 100 µℓ running buffer and applied to the multiplexed nitrocellulose membranes blotted with antibodies for *sodB*, *IpaB* and *IpaH* genes.

Single versus Multiplexed Gene Detection

Individual genes were amplified in a single PCR reaction (i.e. *sodB* gene amplified in a tube containing only *sodB* primers) and the amplified PCR products were detected using agarose gel electrophoresis and lateral flow immunoassay. The PCR products were visualised on agarose gel electrophoresis as this is the gold standard for the visualisation of PCR products. The introduction of lateral flow immunoassay for the qualitative analysis of the same PCR products demonstrated satisfactory results. The individual amplified *sodB* and *IpaB* genes

could be detected on the multiplexed nitrocellulose membranes at the correct antibody positions and did not display non-specific binding or incorrect anti-tag binding (Figure 10a-b). However, when the *IpaH* gene amplified product migrated across the nitrocellulose membrane positive bands were observed at all 3 test line positions (Figure10c). The intensity of the lines varied with the anti-BO (*sodB*) and anti-AF (*IpaB*) lines being less intense than the anti-TxR (*IpaH*) line. This may have been due to possible cross contamination during the antibody blotting as it had only occurred once. All other test strips used for the visualisation of *Shigella* spp. (*IpaH*) products did not yield non-specific binding.

Multiplexed PCR products were analysed using agarose gel electrophoresis and lateral flow immunoassay. A multiplexed PCR product was applied to the multiplexed nitrocellulose membrane and migrated across the membrane. No bands were detected after a 30 minute incubation period at room temperature; on the contrary bands were detected on the agarose gel (Figure 11).

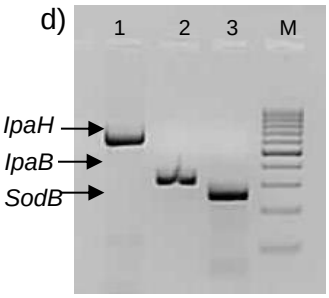
***Vibrio* (*sodB*)**	***Salmonella* (*IpaB*)**	***Shigella* (*IpaH*)**	**Agarose gel**
a) 	b) 	c) 	d) 

Figure 10 Agarose gel and LFIA strips showing the visualisation of single amplified genes of *V. cholerae non* O1, *S. typhimurium* and *S. dysenteriae* with LFIA and gel electrophoresis.

This may have resulted from insufficient SCG for colour production or competition among the amplified genes to bind to the SCG and migrate at a steady velocity to the test line region. It was evaluated by amplifying various combinations of genes and subjecting them to the multiplexed nitrocellulose membrane to assist in determining which genes could possibly be outcompeting each other. The amplification tube combinations were ❶ *V. cholerae* non O1 and *S. typhimurium*, ❷ *S. typhimurium* and *S. dysenteriae* and ❸ *S. dysenteriae* and *V. cholerae* non O1.

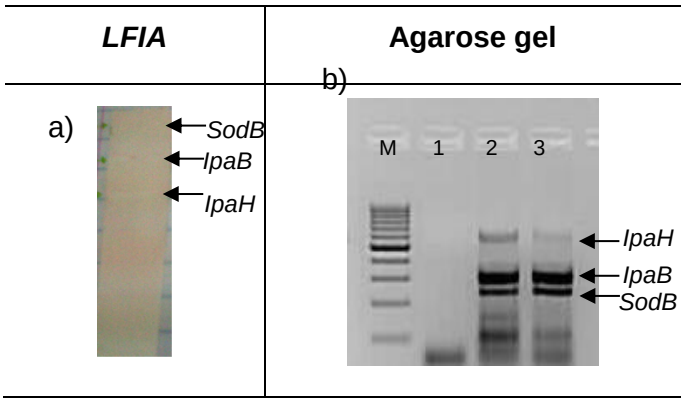


Figure 11 Agarose gel and multiplexed LFIA strip showing the visualisation of a multiplexed amplified PCR for genes of *V. cholerae non* O1, *S. typhimurium* and *S. dysenteriae* with LFIA and gel electrophoresis.

Positive bands were observed for the amplified genes in tube 1 (*V. cholerae* and *S. typhimurium*) and tube 2 (*S. typhimurium and S. dysenteriae)* (Figure 12). Corresponding test lines were detected and no non-specific binding occurred. From the image in Figure 12c no bands were detected in tube 3 which contained *S. dysenteriae* and *V. cholera* non O1 amplified genes. The inhibition of the multiplexed sample may have been caused by the combination of *S. dysenteriae* and *V. cholerae* in a single tube. A feint band at the *sodB* region on the agarose gel was observed thus the gene was amplified.

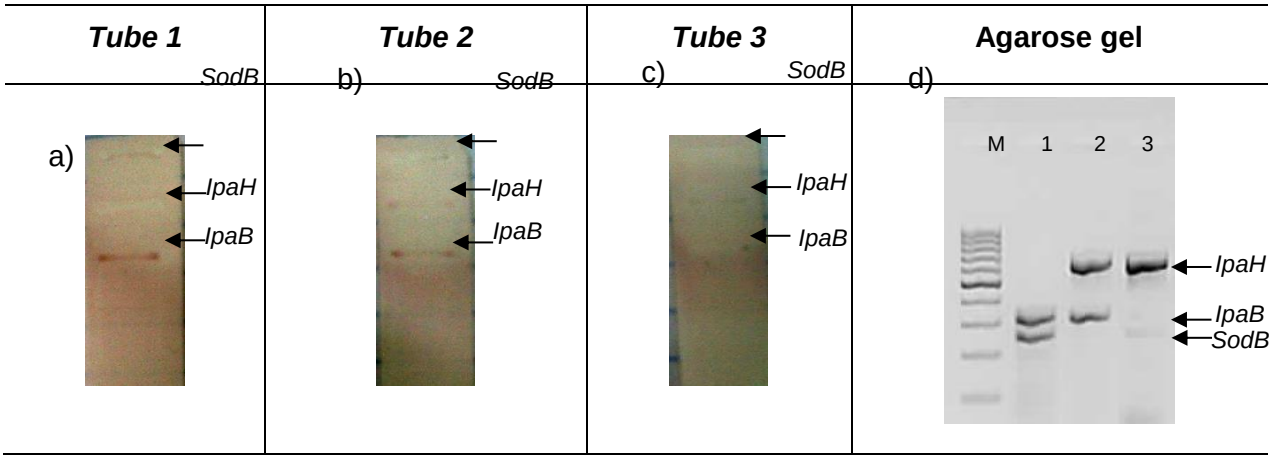


Figure 12 Agarose gel and LFIA strips showing the visualisation of different gene combinations with LFIA and gel electrophoresis.

It was concluded that a multiplex PCR amplification of genes would not be suited to obtain accurate results when using the lateral flow immunoassay test strips. Subsequently the multiplexed lateral flow immunoassay test strips were optimised for the detection of single amplified PCR products only.

4.3 VALIDATION OF LATERAL FLOW IMMUNOASSAY

Validation of new methods or techniques is required prior to clinical or laboratory use to ensure that they meet quality control and regulatory requirements among other criteria (Chan, 2008). Development of the lateral flow immunoassay was validated in terms of sensitivity, specificity, reliability, precision and accuracy. These parameters were selected to obtain sufficient information on the suitability of this method for its intended use prior to undertaking a full validation process.

Specificity of the lateral flow immunoassay test strip was determined by assessing its capability to produce a positive result for only the selected genes which were of critical importance for multiplex detection (Li et al., 2013). Twenty-seven bacterial reference strains were amplified with the labelled primer PCR and visualised with both the LFIA and agarose gel electrophoresis. Strains included commensal and pathogenic *E. coli* strains, *Shigella*-, *Salmonella*-, *Vibrio* species and other strains of the *Enterobacteriaciae* family (Table 11). Figure 13 shows a comparison of the results obtained for *Vibrio* spp. using both the agarose gel electrophoresis and lateral flow immunoassay.

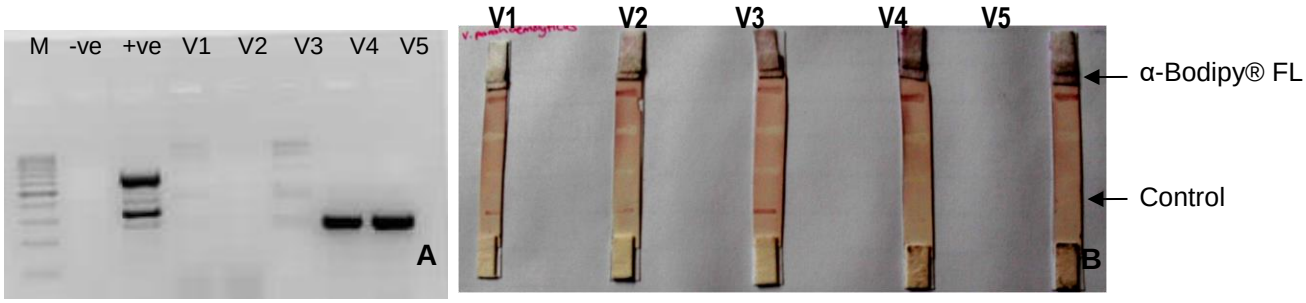


Figure 13 Specificity study for selected genes (*sodB*-*Vibrio* spp.; *IpaH*- *Shigella* spp. & EIEC; *IpaB*- *Salmonella* spp.).Shown is a direct comparison of A) agarose gel electrophoresis and B) lateral flow immunoassay for **V1**-*V.paraheamolyticus*; **V2**-*V. fluvialis*; **V3**-*V. cholerae* non O1; **V4**-*V. cholerae* O1 and **V5**-*V. cholerae* O139.

The lateral flow immunoassay was specific and no cross reactivity for amplified PCR products of non-specific strains was detected on the test strips. The 5 *Vibrio* spp. samples demonstrated a band at the sodB gene antibody region of the LFIA test strip. Bands were not detected on agarose gel electrophoresis but produced bright red bands on the lateral flow immunoassay. It was therefore noted that the LFIA was highly specific and more sensitive than the routinely used agarose gel electrophoresis. A control line was present in all test strips thus affirming the validity of the results obtained. Band intensities varied among test strips and began to fade somewhat after the test strips were read. When strips were photographed in some instances

the control line was not visible because of the low resolution of the image but was visible on the actual strip. The possibility of low control line intensity can be explained by competitive binding of the amplicons to the labelled colloidal gold particles and the streptavidin anti-mouse IgG at the control line (Anfossi et al., 2010). Genes coding for *Vibrio*-, *Salmonella*-, *Shigella* spp. and EIEC were correctly identified using lateral flow immunoassay strips thus confirming the specificity of the LFIA. EIEC produced an *IpaH* gene band on the gel electrophoresis and the amplicons were detected using the lateral flow immunoassay strip thus confirming its ability to detect EIEC.

Table 11 Bacterial strains used to test the specificity of the lateral flow immunoassay

Bacterial strain	Source	*IpaB*	*IpaH*	*sodB*	Control
Commensal *E. coli*	NHLS	✗	✗	✗	✓
Enteroaggregative *E. coli*	NHLS	✗	✗	✗	✓
Enteropathogenic *E.coli*	NHLS	✗	✗	✗	✓
Enterohaemorrhagic *E.coli*	NHLS	✗	✗	✗	✓
Enterotoxigenic *E.coli*	NHLS	✗	✗	✗	✓
Enteroinvasive *E.coli*	NHLS	✗	✓	✗	✓
S. dysenteriae type 1	NHLS	✗	✓	✗	✓
S. boydii serotype B	NHLS	✗	✓	✗	✓
S. flexneri	NHLS	✗	✓	✗	✓
S. sonnei	NHLS	✗	✓	✗	✓
V. cholerae non-O1	NHLS	✗	✗	✓	✓
V. cholerae O1	NTCC	✗	✗	✓	✓
V. parahaemolyticus	NCTC	✗	✗	✓	✓
V. cholerae O139	NHLS	✗	✗	✓	✓
V. fluvialis	NCTC	✗	✗	✓	✓
S. typhi saltyO1	NHLS	✓	✗	✗	✓
S. typhi salty O2	NHLS	✓	✗	✗	✓
S. typhimurium SaltmO1	NHLS	✓	✗	✗	✓
S. typhimurium SaltmO2	NHLS	✓	✗	✗	✓
S. paratyphi	NHLS	✓	✗	✗	✓
S. paratyphi B	NHLS	✓	✗	✗	✓

S. paratyphi C	NHLS	✓	×	×	✓
S. enteritidis	NHLS	✓	×	×	✓
Klebsiella pneumoniae	NHLS	×	×	×	✓
Ent. Faecium	NHLS	×	×	×	✓
Ent. Faecalis	NHLS	×	×	×	✓
M. morganni	NHLS	×	×	×	✓

✓: Band detected

×: No band detected

The precision of the assay was determined by measuring the repeatability and reproducibility of the data using the electrophoresis as the reference method. Repeatability is determined by measuring the data of the method obtained during a single analytical run (i.e. same method run on identical test material under the same conditions). Repeatability of the lateral flow immunoassay was validated by analysing triplicate amplicons for each of the virulence genes with a single batch of lateral flow immunoassay strips on one specific day (Figure 14).

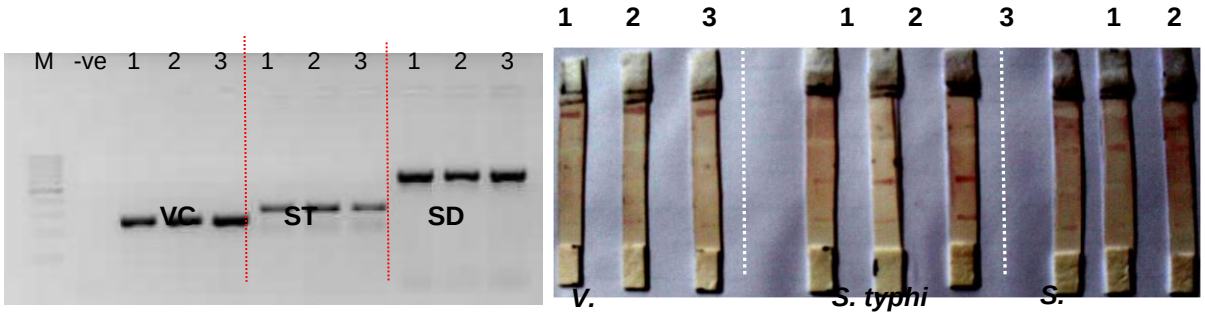


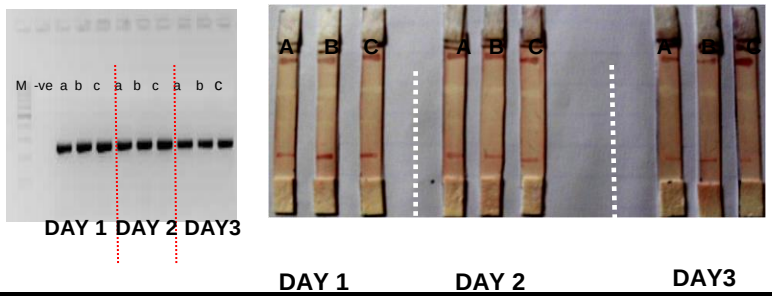
Figure 14 Agarose gel and LFIA strips showing the repeatability of the assay for *V. cholerae*, *S. typhi* and *S. dysenteriae*. Identical results were obtained when triplicate PCR amplified products were analysed on the same day under standard conditions.

Positive bands were obtained for the triplicate amplicons of *V. cholerae*, *S. typhimurium* and *S. dysenteriae* at the correct positions on the test strips. The data correlated with the genes visualised on agarose gel electrophoresis. Band intensities varied slightly, however the presence of a pink/red line was read as positive. From the results obtained lateral flow immunoassay test strips were deemed repeatable in terms of detecting the same amplified genes from three individual samples analysed in triplicate under standard conditions.

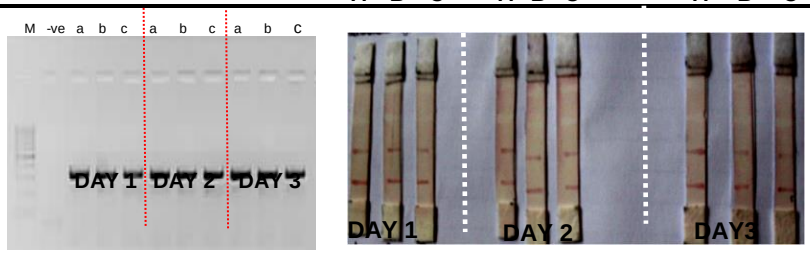
The reproducibility study was devised such that triplicate samples were analysed for the three organisms of interest on three different days using a different batch of lateral flow immunoassay strips on each day (3x3x3). Figure 15 demonstrates that the test and control lines obtained for each sample was consistent for all three organisms. It can thus be concluded

that the lateral flow immunoassay can be reproduced at any given time and would display the same result (presence/absence).

A)	*V 1*	*V2*	*V3*
Day 1	✓	✓	✓
Day 2	✓	✓	✓
Day 3	✓	✓	✓



B)	*ST 1*	*ST2*	*ST3*
Day 1	✓	✓	✓
Day 2	✓	✓	✓
Day 3	✓	✓	✓



C)	*SD 1*	*SD2*	*SD3*
Day 1	✓	✓	✓
Day 2	✓	✓	✓
Day 3	✓	✓	✓

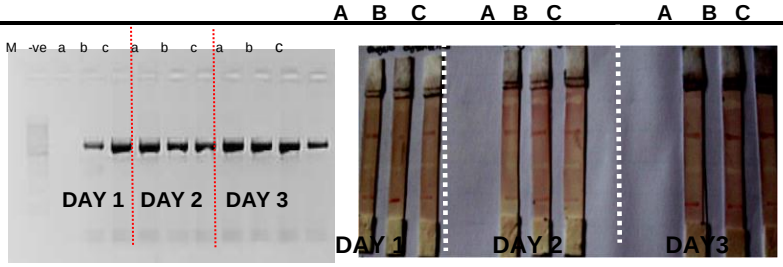


Figure 15 Agarose gel and LFIA strips showing the reproducibility of the assay for A) *V. cholerae,* B) *S. typhi* and C) *S. dysenteriae*. Identical results were obtained when triplicate PCR amplified products were analysed on three consecutive days.

The sensitivity of an assay was determined by analysing serially diluted amplicons of each gene in three repeats. The limit of detection (LOD) was defined as the complete disappearance of red colour at the relative test line position. The initial DNA concentrations added into the PCR tube prior to amplification were as follows; 2.40 µg/ml of *S. typhimurium* DNA, 0.207 µg/ml of *V. cholerae* DNA and 1.56 µg/ml of S. *dysenteriae* DNA. During DNA extraction the entire broth was used for the isolation of *Salmonella* and *Shigella* spp., whereas

for *V. cholerae* only the surface growth could be used as the broth cannot be shaken thus a lower *V. cholerae* DNA concentration was obtained. The logarithmically amplified DNA concentrations were not quantified as the DNA quantification kit could not detect at a concentration greater than 100 µg/ml. Thus, the actual concentrations (amplified logarithmically) in the PCR tubes were not known and as such the LOD was determined at the lowest detectable dilution.

As highlighted in Table 12 the LOD of the lateral flow immunoassay test strip for *V. cholerae, S. typhimurium and S. dysenteriae* were 16, 8 and 8 times serially diluted, respectively. The lateral flow immunoassay thus demonstrated suitable sensitivity in terms of detecting diluted amplified samples. The test strips were designed to detect logarithmically amplified PCR products without further dilution thus the sensitivity criteria was achieved.

Table 12 Determining the sensitivity and LOD of LFIA by the detection of various concentrations of *V. cholerae* non O1, *S. dysenteriae* and *S. typhimurium*

Test bacterium	Immobilised antibody	Label	Amplified PCR Dilutions						
			Neat	2x	4x	8x	16x	32x	Neg
V. cholerae	Anti-BOdipy® FL	BOdipy® FL	S+ve	S+ve	S+ve	M+ve	W+ve	-ve	-ve
S. typhimurium	Anti- alexa fluor 405	Cascade blue	S+ve	S+ve	M+ve	W+ve	-ve	-ve	-ve
S. dysenteriae	Anti- Texas red	Texas red	S+ve	S+ve	S+ve	M+ve	-ve	-ve	-ve

S+ve: 3 replica positive results
M+ve: 2 replica positive and 1 negative result
W+ve: 2 replica negative and 1 positive result
-ve: 3 replica negative results

4.4 FIELD EVALUATION

A pilot study using extracted DNA from an array of previously assayed environmental samples was conducted in a laboratory setting. In total, 138 samples consisting of positive (*n*=109) and negative (*n*=29) samples were subjected to PCR amplification and visualisation using the LFIA test strip and gel electrophoresis. The previously analysed data will be referred to as initial analysis data and was used as comparison for the LFIA results.

During the initial analysis the DNA was extracted from the samples using the commercial DX Universal Liquid Sample DNA extraction kit (Corbett Life sciences) and a guanidinium thiocyanate DNA extraction method as adapted from Omar and Barnard (2014). The DNA was amplified using species specific multiplex PCR (m-PCR) assays for *E. coli*, *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. The different DNA extraction methods employed demonstrated the efficacy of the LFIA test strip to produce tangible results irrespective of the type of extraction method used. The samples which produced positive bands during the species specific m-PCR assays were subjected to amplification with the relevant single gene labelled primers for the gm-PCR assay. Random labelled primers were used for the amplification of the control samples. The amplicons were simultaneously applied onto agarose gel electrophoresis and LFIA strips. The data obtained from both assays were analysed and compared to the known initial analysis data in terms of the LFIA capability to show presence/absence of the amplified genes (Table 13).

Thirty *Vibrio* spp. positive samples were analysed for the *Vibrio* spp. housekeeping gene (*sodB*). On gel electrophoresis the 248bp gene was visualised in 11 (37%) samples whereas 28 (93%) samples produced a positive test line for the *sodB* gene on the LFIA strips. The initial results were more accurately replicated with the LFIA whereas only a third of the data was replicated using gel electrophoresis. This may be as a result of better sensitivity of the lateral flow immunoassay over the reference method.

From the 46 samples tested for the *IpaB Salmonella* housekeeping gene, the 314bp gene was present in 22 (48%) samples on gel electrophoresis and 14 (30%) samples on the LFIA strips. However of the 14 samples, seven were invalid as the control line was absent. Hence they could not be regarded as true positives. After exclusion of the invalid strips, 7 (15%) samples were read as positive for *IpaB* on the LFIA strips. The poor detection of the IpaB gene on LFIA may have been due to various factors during the manufacture process and reagent preparation for this specific batch of test strips (Wild et al., 2013). Possible degradation of the stored extracted DNA after the initial study phase could also have resulted in the poor replication of data (Sajid et al., 2015).

A total of 62 samples were analysed for the *IpaH Shigella*/EIEC housekeeping gene. As shown in Table 13 from the initial analysis, the *IpaH* gene was present in 33 samples only, however for this study all 62 (29 control) samples were analysed on both the LFIA and gel electrophoresis. Ten (16%) samples contained the *IpaH* gene on gel electrophoresis and 5 (8%) samples displayed a positive test line on LFIA strips.

Table 13 Comparison of gel electrophoresis and Lateral Flow Immunoassay (LFIA) qualitative results for environmental samples

Pathogen	***N***	***Positive results by:***		
		Initial analysis	**Agarose gel electrophoresis**	**LFIA**
Vibrio (*sodB* gene)	30	30	11	28
Salmonella (*IpaB* gene)	46	46	22	14
Shigella/ EIEC(*IpaH* gene)	62	33	10	5
	138	**109 (79)**	**43 (31)**	**47 (34)**

n: Number of samples analysed; (): percentage

Three samples tested positive for the *IpaH* gene on gel electrophoresis and likewise for the LFIA which were negative during the initial analysis. This may either have been faintly positive on gel electrophoresis during the initial analysis or the DNA was not concentrated enough and thus was undetectable during the initial analysis study. The second theory may be valid as the samples were collected from wastewater which is known to be highly concentrated with a mixture of bacterial, viral and fungal pathogens. For the application and detection on LFIA the DNA was amplified using tagged primers specific for the *IpaH* gene and thus the amplified DNA may have enabled easier detection on LFIA.

A complete analysis of the samples tested as shown in Table 13 demonstrated that 43 and 47 samples were positive on gel electrophoresis and LFIA respectively from a total of 109 previously screened known positive samples. Therefore, regardless of the detection methods used 83% of the initial results were reproducible.

Comparison of the 29 control samples analysed, 24 (83%) samples were in agreement for all three methods (Table 14). One sample displayed a band on both the agarose gel and the lateral flow immunoassay therefore the data was not discarded. This could have resulted from either human error during the initial analysis, recorded incorrectly or possible contamination during the re-testing process. The remaining four samples were concluded to have been false positives on either the agarose gel electrophoresis or lateral flow immunoassay. To rule out

this possibility in future studies such samples will be tested in triplicate and the majority result should be reported.

Table 14 Overall analysis of the control samples result combinations for the three detection methods used

N=29 (%)	***Result combinations***		
	Initial analysis	**Agarose gel electrophoresis**	**LFIA**
24 (83)	-	-	-
2(7)	-	+	-
1(3)	-	+	+
2(7)	-	-	+

Despite these shortfalls it can be concluded that the LFIA performs well in comparison to the gel electrophoresis in terms of sensitivity. No false positives were detected using the LFIA hence the test strips are capable of producing statistically reliable results. The advantage of LFIA over gel electrophoresis is its ease of use and the reduced time required to obtain a result. The test strips can be easily manufactured using automated systems once optimised and can be stored at ambient temperature prior to use, however, agarose gels need to be freshly prepared and used immediately.

Although the lateral flow immunoassay was tested with environmental samples available in the laboratory an on-site end user study will offer greater insight into the true success of this assay. The data shows that the lateral flow immunoassay test strips could detect and differentiate correctly between the *Vibrio*-, *Salmonella*-, *Shigella* spp. and EIEC from the various environmental samples. The test strips will require some additional modifications prior to its implementation in routine and point of use analysis; however the main objective of the study was achieved in that a multi-analyte lateral flow immunoassay was designed for the onsite detection of selected bacterial pathogens from various sources.

4.5 FINAL CONSIDERATIONS

The last considerations for this project was the estimated cost for the test strip and test, including the housing unit for the LFIA. Based on the expenses during the experimental work the estimated cost for the laboratory production and running of the LFIA was R100 and

excluded the housing unit. It is believed that as the production volumes increases, and larger quantities of the consumables are bought, this cost may be reduced.

The housing unit could not be produced from plastic but a 3D printed version of the housing unit was constructed (Figure 16). It was developed to house the strip dimensions given in Table 10 and fitted the test strip perfectly. The two section assembles easily and will only be produced and tested in a final version once the decision to produce the test strip has been made.

Figure 16 A schematic (left) and 3D printed versions of the test strip housing unit showing the separated (middle) and combined (right) housing unit with the test strip.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 INTRODUCTION

The aim of the study was to investigate the possibility of developing a portable alternative method for the visualization and confirmation of amplified gene fragments. Newer technologies such as nucleic acid lateral flow immunoassays provide a simplified molecular detection option. With this in mind a proof of concept study was designed to explore the possibility of using the LFIA principle for the detection of *Salmonella*-, *Shigella*-, *Vibrio*- and EIEC species in conjunction with a conventional PCR assay already employed for the amplification of the above-mentioned pathogens in a laboratory setting. This study focused on the potential use of the LFIA as an alternative to gel electrophoresis because of its ease of use, rapidity and reliability.

5.2 CONCLUSIONS

According to the results presented the LFIA was successfully designed and developed for the detection of *Salmonella*-, *Shigella*-, *Vibrio*- and EIEC species from various sample types. The assay was specific in terms of detecting only the specific pathogens and could be correlated with the results visualised on an agarose gel. The data obtained during the validation study indicated that the LFIA was reliable and the results could be reproduced. The LFIA test strips displayed good sensitivity for the detection of all selected pathogens and therefore demonstrated that a multiplex LFIA can successfully detect and produce results rapidly for the presence of *Salmonella*-, *Shigella*-, *Vibrio*- and EIEC species.

This proof of concept LFIA can be used as a screening assay with the use of minimal equipment, making it an ideal system for point of care or field use. However there were hindrances during the development process and would need to be improved prior to implementation.

5.3 RECOMMENDATIONS

The following shortfalls were identified during the study and needs to be investigated to improve the LFIA test strip prior to implementation and possible commercialisation:

- Inclusion of the *E. coli* housekeeping gene onto the test strip for the screening of *E. coli* commensal and pathotypes.

- The *Salmonella* positive samples produced extremely feint bands on the LFIA thus the rate of release of gold nanoparticles will be investigated for improved detection of the *Salmonella* gene.
- The test strips were manufactured and used for analysis within 2-3 days thus studies to determine optimal storage times and shelf life of the test strips need to be conducted.
- Currently the commercially available housing cassettes are designed for single product detection thus a housing cassette suitable for multiplexed product detection will be designed.
- Once the abovementioned factors have been rectified a complete validation in terms of inter-laboratory validation needs to be conducted.

CHAPTER 6
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