Development of two rapid diagnostic tests for the detection of Bovine Salmonellosis in calves with scour

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Abstract

Salmonellosis is a worldwide issue, that impacts human and animal health alike. Infection is often derived from foodborne contamination, causing gastroenteritis and in extreme cases, bacteraemia, and death.

Current diagnostics for the detection of *Salmonella* sp. can take a minimum of three days. However once infected with *Salmonella enterica* serovar Dublin (*S.* Dublin), calves often die within 48 hours. Salmonellosis in calves is associated with scour, however it is not the only aetiological agent of diarrhoea in cattle. Antibiotics for potential salmonellosis are often administered before a definitive diagnosis is given, to reduce animal suffering and mortality rates. However, with the emergence of multi-drug resistant strains of *Salmonella* sp. efforts need to be made to ensure antibiotics are only prescribed when bacteria are the causal agent of infection.

Rapid detection methods for pan-*Salmonella* are needed to prevent calf death and enable targeted treatment. This would reduce the impact of the disease on animal welfare, as well as to safeguard public health, reduce economic impacts, and enable the right treatment is prescribed for the right disease

In this study two rapid diagnostic methods were developed: a nucleic amplification assay targeting *Salmonella* DNA known as loop-mediated isothermal amplification (LAMP), and a potentiometric immunoassay targeting surface antigens on *Salmonella* bacteria using biosensors in the Vantix System. Both diagnostics were found to be rapid and robust, with high sensitivity and specificity to multiple *Salmonella* serovars. Fluorometric LAMP assays detected pan-*Salmonella* in 35 minutes, with visualisation under a UV light. Potentiometric immunoassays on the Vantix reader 2.0, were able to detect *S*. Dublin through undiluted calf scour in under an hour. Both diagnostic methods would enable rapid detection of *Salmonella* sp. in calves suffering from scour.

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"Without you I would never be me, you are the leaves of my family tree"

Sing Together – Train

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List of Abbreviations:

A99H	Thermofisher monoclonal antibody (A99H)
AHDB	Agriculture and horticulture development board
APHA	Animal and Plant Health Agency
App.	Appendix
AMR	Anti-microbial resistance
B3	Reverse outer primer
BGA	Brilliant green agar
BHI	Brain-heart infusion
BIP	Reverse internal primer
BLAST	Basic Local Alignment Search Tool
BLoop	Reverse loop primer
BMM	Bio-rad mouse monoclonal antibody (5D12A)
bp	Base pairs
BP	Reverse primer
BRP	Bio-rad rabbit polyclonal antibody
CDS	Coding sequence
CFU/ml	Colony-forming unit per millilitre
DNA	Deoxyribonucleic acid
Dream *Taq*	Dream *Taq* Green master mix
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immune-sorbent assays
F3	Forward outer primer
FIP	Forward internal primer
FLoop	Forward loop primer
FP	Forward primer
g	Grams
GC	Guanine-cytosine
g/l	Grams per litre
HotStarTaq	HotStarTaq Plus master mix
hr	Hour
HRP	Horseradish peroxidase
I. D	Identification
L	Litre
LAMP	Loop-mediated isothermal amplification
LCB	Locally colinear blocks
LL	Lightning-link
M	Moles
MC	Multi-channel pipette
MDR	Multi-drug resistant
min	minutes
mg	Milligram
Mg^{2+}	Magnesium ion
ml	Millilitre

N	Nucleotide
NA	Nutrient agar
NCBI	National centre for biotechnology information
NCTC	National collection of type-cultures
ng	Nanogram
nm	Nanometre
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE V.4	PrimerExplorer V.4
PHE	Public Health England
PI	Propidium iodide
RIfS	Reflectometric interference spectroscopy
RNA	Ribonucleic acid
rpm	Rotations per minute
rRT-PCR	Real-time reverse transcription PCR
RT-PCR	Real-time PCR
S.	*Salmonella enterica* serovar
SS	SYBR safe
SEM	Scanning electron microscopy
TBE	Tris/Borate/EDTA buffer
TEM	Transmission electron microscopy
Tm	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
TRP	Thermofisher Rabbit Polyclonal antibody
UDG	Uracil-DNA glycosylase
UK	United Kingdom
UV	Ultraviolet
v/v	Volume to volume
Vol	Volume
VR1	Vantix research tool
VR2	Vantix research tool 2.0
WB	Wash bottle
w/v	Weight to volume
XLD	Xylose-lysine-deoxycholate agar
ΔG	The change in free energy
µl	Microlitre
µm	Micrometre

1. Introduction

1.1 Introduction to *Salmonella* sp.

Salmonellae are facultative anaerobic, non-spore forming Gram-negative rods belonging to the family of *Enterobacteriaceae* (Mastroeni *et al*., 2000; Felix and Angnes, 2018; Silva *et al*., 2019). These zoonotic pathogens play an important role in foodborne disease and have a broad host range, *Salmonella enterica* has been isolated from multiple animal species, including mammals, birds and reptiles (Nielsen, 2012; Costa *et al.*, 2012; Adhikari *et al.*, 2009; Lomborg *et al.*, 2007; Biswas *et al.*, 2010; Cheung and Kam, 2012; Yang *et al*., 2016 Mastroeni *et al*., 2000; Filioussis *et al*., 2008; Switt *et al*., 2009). Ubiquitous in the environment, *Salmonella* sp*.* can multiply outside of the host in moist warm conditions, survive for long periods in organic matter such as stored slurry, cattle manure and soil, as well as survive for years in dried-in faecal matter (Plym-Forshell and Ekesbo, 1996; Taylor and Burrows, 1971; Wray and Davies, 2000).

Salmonella sp. are commonly identified via serotyping, biochemical profiling and phage typing (Wattiau *et al.*, 2011). The Kauffman-White scheme is a widely used serotyping method for strain differentiation and sub-typing of *Salmonella* (Switt *et al.,* 2009). Routinely performed for over 80 years and based on antigenic variability, the method uses agglutination reactions of *Salmonella* sp. with specific antibodies to identify surface antigens (Switt *et al.,* 2009; Wattiau *et al.*, 2011). This determines the antigenic makeup of the organism, identifying lipopolysaccharides (O antigen), flagellar proteins (H antigen), and capsular polysaccharides (Vi antigen) present on the surface of the cell (Switt *et al.,* 2009; Wattiau *et al.*, 2011).

Within the genus *Salmonella*, there are two species; *Salmonella enterica* (*S. enterica*) and *Salmonella bongori*, with the number of serovars per species at 2557 and 22 respectively (Table 1.1; Grimont and Weill, 2007). *S. enterica* is divided into six subspecies (Table 1.1). *S. enterica* ssp. *enterica* is associated with warm-blooded animals, such as humans and cattle, with serovars including *Salmonella enterica* serovar Dublin (*S.* Dublin) and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium; Moore and

Feist, 2007). Humans suffer from salmonellosis worldwide: due to the large amount of *Salmonella* serovars, it is important to identify the causal strain of salmonellae to identify the source of infection.

Table 1.1 The number of serovars within each subspecies of *S. enterica.* (Data taken from Grimont and Weill, 2007).

***S. enterica* subspecies**	**Number of serovars per subspecies**
arizonae	99
diarizonae	336
enterica	1,531
houtenae	73
indica	13
salamae	505
Total serovars within *S. enterica*	2,557

Historically 'O' antigen groups were designated letters but as letters ran out, 'O' groups were denoted with numbers. Grimont and Weill (2007) report an update in the nomenclature and serotype coding for the Kauffman-White scheme (See Table 1.2). They present a new designation system, with 'O' groups being described by their somatic 'O' factors and advise abandoning letter designations. Both letter and number designations are currently used, thus when 'O' groups are referred to within this study, current nomenclature will be used, with letter designations in brackets for ease of use, for example; O:4 (B), O:6, 14 (H), O:1, 3, 19 (E_4).

Switt *et al.* (2009) note that *Salmonella* sp. can be motile through peritrichous flagella ('H' antigen), a characteristic that is regulated through 'phase variation'. On the bacterial chromosome, flagella are encoded by two different genes (*fliC* and *fliB*), the majority of *Salmonella* sp. are biphasic and can express both genes. However, some *Salmonella* sp. are monophasic, lacking one of the *fli* genes, and

can be non-motile, such as *S.* Dublin (Switt *et al*., 2009). By understanding the genes and proteins expressed by *Salmonella* serovars, we can utilise them as targets for detection of salmonellae.

Table 1.2: Historical 'O' group nomenclature with the current 'O' group nomenclature as suggested by Grimont and Weill (2007). 'O' groups within this study are referred to with current nomenclature and letter designations in brackets for ease of use. (Data taken from Grimont and Weill, 2007).

Historical 'O' group nomenclature	**Current 'O' group nomenclature**
A	2
B	4
C_1-C_4	6, 7
C_2-C_3	8
D_1	9
D_2	9, 46
D_3	9, 46, 27
E_1-E_2-E_3	3, 10
E_4	1, 3, 19
F	11
G_1-G_2	13
H	6, 14
I	16
J	17
K	18
L	21
M	28
N	30
O	35
P	38
Q	39
R	40
S	41
T	42
U	43
V	44
W	45
X	47
Y	48
Z	50

1.2 Salmonellae in human health

A bacterium of worldwide importance, infections with *Salmonella* sp. represent a serious medical and veterinary problem, with non-typhoid *Salmonellae* among the leading causes of food-borne disease in humans, making it a significant concern for food and feed safety (Mastroeni *et al.,* 2000; Farrell *et al*., 2005; Filioussis *et al.,* 2008; Pullinger *et al*., 2010; Yang *et al*., 2016; Silva *et al*., 2019).

Typhoid fever is caused in humans by *Salmonella enterica* serovar Typhi (*S.* Typhi), which is host-adapted to humans and often results in systemic infection (Villarreal-Ramos *et al*., 2000). Within many developing countries, typhoid fever is still endemic often due to human consumption of faecal-contaminated water (Mastroeni *et al*., 2000).

Humans are quite susceptible to gastroenteritis, with an elevated incidence in many countries caused by foodborne pathogens such as *Salmonella* sp., *Campylobacter coli,* and *Escherichia* coli; contaminated foods can cause serious disease in animals and humans, as well as accelerated food spoilage (Eng *et al*., 2015; Felix and Angnes, 2018). People infected with acute salmonellosis can develop fever, vomiting, diarrhoea and abdominal cramps (Felix and Angnes, 2018; Wang *et al*., 2018). The potential zoonosis of *Salmonella* can cause severe invasive infections within susceptible humans, such as the immunocompromised, resulting in hospitalisation (Nielsen *et al.*, 2004; Vo *et al.*, 2006; Mateus *et al.*, 2008; Wang *et al*., 2018).

Costa *et al.* (2012) suggested that cattle are one of the most common sources of infection for human salmonellosis, which is one of the most important foodborne diseases worldwide and has a significant impact on public health (Jadidi *et al.*, 2012; Costa *et al.*, 2012; Biswas *et al.*, 2010; Wu *et al.*, 2014). Salmonellosis in humans is commonly associated with foodborne transmission: a study by Cummings *et al.* (2012) showed that, once food exposures were controlled for, direct contact with dairy cattle or their environment was significantly associated with salmonellosis caused by bovine-matched subtypes. Hoszowski and Wasyl (2000) suggest that most human salmonellosis cases are traced to

Salmonella infected farm animals. Due to zoonosis, those working in close contact with cattle are at a greater potential risk for *Salmonella* transmission which could result in additional economic and welfare issues if not properly controlled (Cummings *et al*., 2012; Switt *et al*., 2009). Yang *et al*. (2016) note that to reduce *Salmonella* outbreaks, a multifaceted approach from farm to table is required to reduce illnesses associated with food products.

1.3 Infection with *Salmonella* sp.

Infection with *Salmonella* sp. is commonly through the faecal-oral route. After ingestion a proportion of the bacteria resist the low pH of the stomach and colonise the mucosa of the terminal ileum and colon, replicating in the sub-mucosa and associating with the M-cells of Peyer's patches (Thiennimitr *et al*., 2011; Brumell *et al*., 2002; Mastroeni *et al*., 2000; Frost *et al.,* 1997; Carter and Collins, 1974).

Specialised epithelial cells such as M cells are involved in initiating an immune response by transporting antigens from the lumen to cells within the immune system (Figure 1.1A; Gebert *et al*., 1996; Corr *et al*, 2007). Upon uptake into intestinal M cells*,* several antigens produced by salmonellae meditate the reorganisation of the cell cytoskeleton of the cells, resulting in membrane ruffling which is the modification of the microvillus structure that enables the bacteria to penetrate the epithelial layer (Figure 1.1B; Frost *et al.,* 1997). Membrane ruffling causes microvilli of the M-cells to disappear in favour of lamellipodia, with which *Salmonella* bacteria associate and are taken up (Figure 1.1C; Frost *et al*., 1997). Neutrophils invaginate invading bacteria and are enclosed by attenuated cytoplasm. Within an hour of infection, bacteria are within the lamina propria of the M cells, where inflammation has recruited neutrophil and macrophages to engulf the bacteria (Figure 1.1D and E; Frost *et al*., 1997). Within an acute infection, *Salmonella* bacteria go no further than the lamina propria (Frost *et al*., 1997). Gastroenteritis symptoms occur within 24 hours of infection: mesenteric lymph nodes are enlarged, and the terminal ileum is congested and distended with fluid, resulting in abdominal pain and diarrhoea (Santos *et al.*, 2001).

With non-host adapted strains, acute infection does not escalate beyond this point, as commonly seen with *S.* Typhimurium infection. With host-adapted strains, such as *S.* Dublin, chronic infection can occur as *Salmonella* sp. disseminates through the blood and lymphatics, via phagocytosis into macrophages/or other phagocytes (Figure 1.1F). By surviving within the vacuole of phagocytes salmonellae can survive and replicate, escaping host immune defences and potentially spreading to the spleen, liver, bone marrow and other tissues (Mastroeni *et al.,* 2000; Brumell *et al*., 2002; Ruby *et al*., 2012; Kurtz *et al.*, 2017).

In healthy adults, ubiquitous serovars (such as *S*. Typhimurium) produce acute but often self-limiting enteritis in diverse species, whilst host-specific serovars (such as *S.* Dublin) are associated with severe systemic disease in a single species, that might not involve diarrhoea, and may cause disease in a limited number of other species (Pullinger *et al*., 2010).

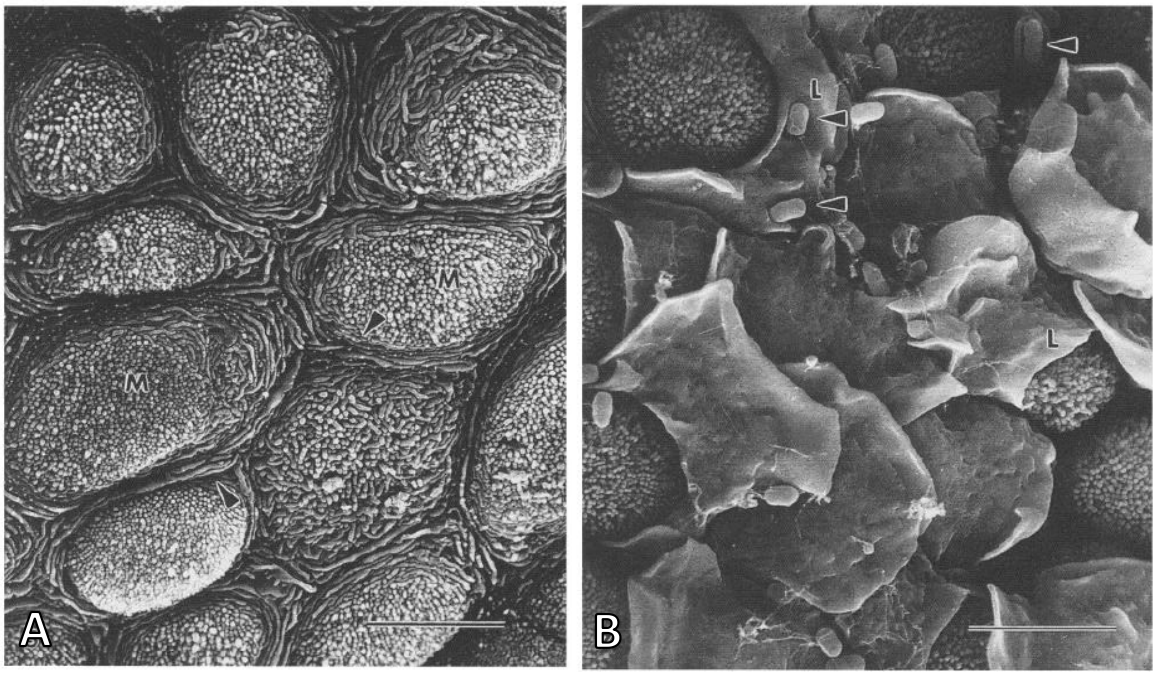

Figure 1.1: Frost *et al*., (1997) use calf ileal loop models to study the initial infection of *S*. Typhimurium within the epithelium of the terminal ileum. SEM = scanning electron microscopy, TEM = transmission electron micrograph

A – SEM of control calf ileum. A group of typical M cells (M) on a domed villus. Characteristic peripheral microfolds (arrowheads) with a central area of short microvilli. Bar = 5µm

B – SEM of calf ileum after exposure to salmonellae for 5 mins. Peripheral folds of the M cells are ruffled, forming Lamellipodia (L), and the bacteria (arrowheads) are being engulfed by the folds. Bar = 5µm

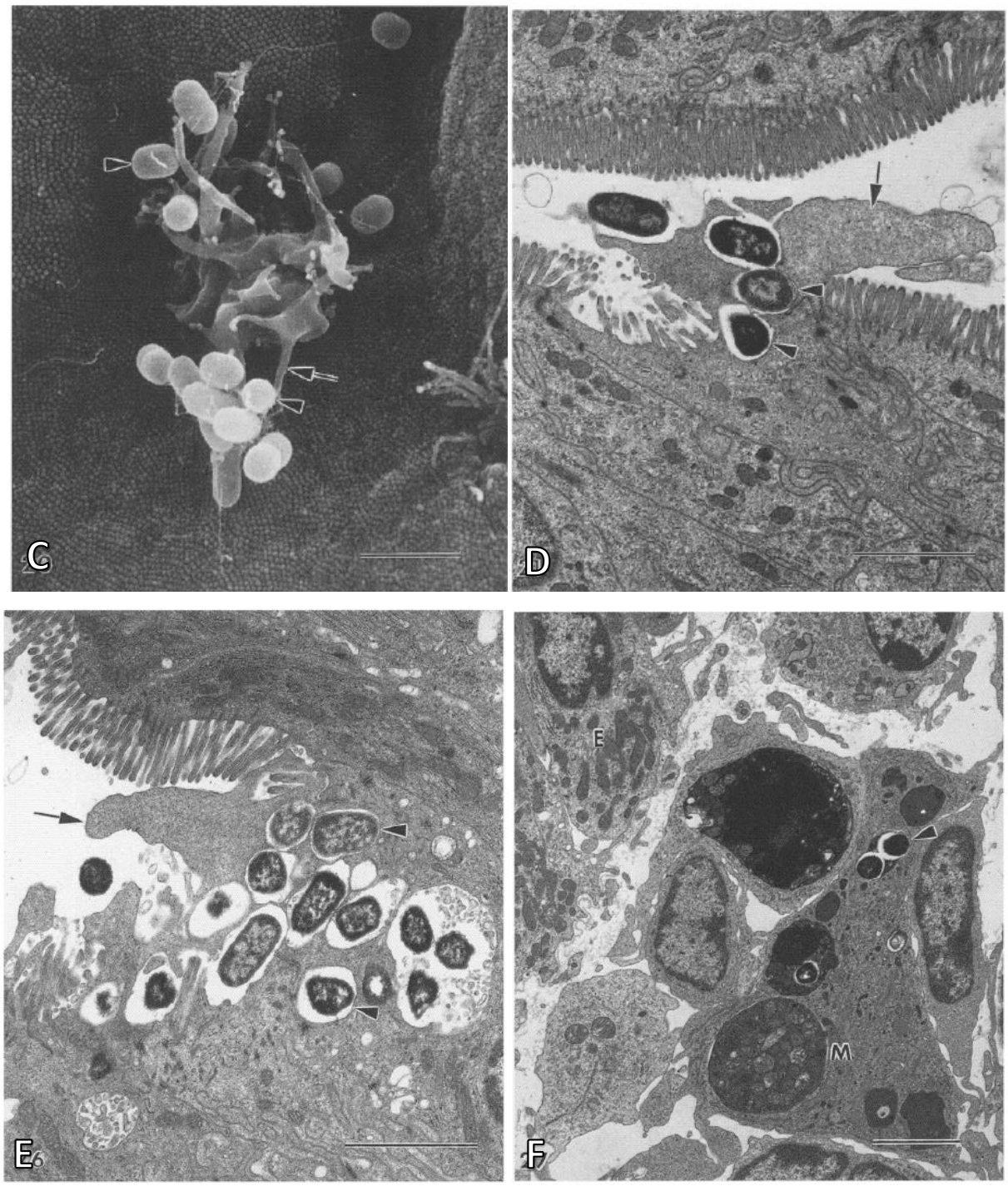

Figure 1.1 continued: Frost *et al*., (1997) use calf ileal loop models to study the initial infection of *S*. Typhimurium within the epithelium of the terminal ileum. SEM = scanning electron microscopy, TEM = transmission electron micrograph

C – SEM of calf ileal Peyer's patch after exposure to salmonellae for 20 mins. Bacteria (arrowheads) are adhering to the lamellipodia and filipodia (arrow) formed at the surface of an enterocyte. Bar = 2µm

D – TEM of calf ileal Peyer's patch after exposure to salmonellae for 20 mins. The surface membrane of the enterocyte has been extensively remodelled into lamellipodia (arrow). Engulfed bacteria are lying in vacuoles (arrowheads). Bar = 2µm.

E – TEM of calf ileal Peyer's patch after exposure to salmonellae for 60 mins. Enterocytes with extensive lamellipodia formation (arrow) are adjacent to normal cells. The cells with lamellipodia contain large numbers of bacteria (arrowheads). Bar = 2µm

F – TEM of calf ileal Peyer's patch after exposure to salmonellae for 60 mins. A macrophage (M) in the lamina propria of an absorptive villus contains membrane-bound bacteria in the cytoplasm (arrowhead), Enterocyte (E). Bar = 2µm

(Pictures from Frost *et al*., 1997)

1.4 Salmonellae in cattle health

Salmonella sp. are commonly associated with infections that result in losses in animal production as well as potential human public health issues due to their zoonotic capability (Vo *et al.,* 2006; Mateus *et al*., 2008). Brumell *et al.* (2002) noted that the outcome of the infection depends on the fitness and genetic complement, the ability of the bacteria to infect and the ability of the host to defend against infection, of both the host and infecting *Salmonella* serovar. Dependent on the strain, cattle can succumb to both enteric and systemic phases of infection (Wallis *et al.,* 1995).

It is recognised that several different *Salmonella* serotypes are associated with bovine salmonellosis; of which are *S.* Dublin and *S.* Typhimurium are commonly reported (Costa *et al.,* 2012; Nielsen, 2012; Nielsen *et al.*, 2007; Zhang *et al.*, 2003; Ruby *et al.*, 2012). Differences are seen with bovine infections with these two serotypes. Cattle infected with *S.* Dublin are more likely to demonstrate long term carriage of the organism rather than transient carriage observed with those infected with *S.* Typhimurium (Santos *et al.*, 2001; Santos & Bäumler, 2004). Additionally, pregnant heifers infected with *S.* Dublin are more likely to abort whilst presenting with limited clinical signs overall (Santos *et al.*, 2001). *S.* Dublin causes significant morbidity in adult cattle whereas a high morbidity and mortality is observed in calves (Rice *et al.*, 1997).

The most important clinical manifestation in calves is diarrhoea, with *S.* Typhimurium causing greater inflammatory and secretory responses, than those observed in *S.* Dublin infection, resulting in an increased acute response (Santos *et al.*, 2001; Wray and Sojka, 1981). However, Santos and colleagues (2001) report that a more invasive infection in calves is observed with *S.* Dublin which can result in various pathological outcomes including polyarthritis, osteomyelitis, meningoencephalitis, and pneumonia.

Historically, *S*. Typhimurium has been the cause of a major salmonellosis epidemic in calves in the UK (Wray *et al*., 1998). Infection with *S*. Typhimurium is often acute, the most affected tissues appear to

be the terminal ileum, colon and cecum; with symptoms which are common with *S*. Dublin infection (Table 1.3; Frost *et al.,* 1997). Villarreal-Ramos *et al.* (2000) noted that whilst *S.* Typhimurium can spread systematically in cattle, systemic organs are not the primary site of bacterial growth, thus in cattle the gut and associated lymphoid tissues are the primary target of *S.* Typhimurium, potentially independent of infection route.

Pullinger *et al*. (2010) noted that host-restricted *S. enterica* must adapt to the changing niches encountered within the host to be able to survive and spread. *S.* Dublin is host adapted to cattle, resulting in a variety of symptoms (Table 1.3; Lomborg *et al.,* 2007). The mechanisms behind host-adaption are not fully understood and in young calves *S.* Dublin is clinically indistinguishable from *S.* Typhimurium (Costa *et al.,* 2012). *S.* Dublin, however, has a much higher potential for systemic dissemination (Costa *et al*., 2012). Whilst host-adapted, the zoonotic potential of *S.* Dublin should not be overlooked, as it can cause invasive infections in humans that are life threatening in susceptible hosts, such as the immunocompromised (Nielsen *et al.*, 2012; Helms *et al.*, 2003; Mateus *et al.*, 2008).

Salmonella Dublin is one of the most prevalent serovars isolated from cattle within Europe and infections in calves continues to be a major problem worldwide (Nielsen, 2013; Vo *et al.*, 2006; Baggesen *et al.*, 2006; Jadidi *et al.,* 2012). Nielsen (2013) noted that *S.* Dublin leads to unacceptable levels of morbidity, mortality and production losses in newly and persistently infected herds. A study by Nielsen *et al.* (2004) showed that calves are often the most commonly infected age group within cattle herds. This susceptibility is due to the calves developing immune system; the production of specific antibodies is less than that seen in older cattle (Da Rogen *et al.,* 1992).

Table 1.3: Infection stages and associated symptoms that can occur in cattle infected with *S*. Dublin (information tableted from Nielsen, 2013). Bacterial shedding can occur through faeces, urine, vaginal discharge, and milk.

Infection Stage	Cattle type affected	Time period since initial infection	Symptoms		Bacterial shedding
			Common	Uncommon/Age Specific	
Peracute	Calves and naïve herds	1-2 days	Bacteraemia followed by endotoxic shock, resulting in death	-	Death occurs before bacteria can be excreted
Acute	All ages	Often 1-3 weeks but can extend to 5-9 weeks	• Enteric infection, which can lead to systemic with transient bacteraemia • Bloody/watery diarrhoea • Depression • Hyperthermia • Loss of appetite	• Calves – pneumonia, arthritis, in rare cases nervous symptoms, often fatal • Adults – abortion, decreased milk production	• Continuous/ intermittent • Large quantity (from 1-10^8 CFU/g)
Chronic	Calves older than 6-8 weeks, after acute infection	Several months	• Failure to thrive • Bloody/loose stool • Shedding of intestinal casts • Slight fever • Scruffy coat • Growth retardation • Lameness due to arthritis/osteomyelitis	• Ischaemic necrosis of skin on ears, tail or distal limbs	May/may not shed bacteria

It has been reported that plasmid-bearing *S*. Dublin strains are highly virulent, resulting in severe enteric and systemic disease with a high mortality in calves (Wallis *et al.,* 1995). Due to plasmid-free strains causing diarrhoea with a low mortality, Wallis *et al*. (1995) proposed that genes on plasmids mediate the persistence of *S*. Dublin at systemic sites. The plasmid did not influence extracellular growth or expression of major outer membrane proteins suggesting it was not involved in the enteric phase of infection nor systemic dissemination (Wallis *et al*., 1995).

Some researchers note that whilst previous *S*. Dublin infection provides some immunity upon re-infection, re-infected animals typically show fewer clinical signs and excrete lower numbers of bacteria (Nielsen, 2013). Hansen *et al*. (2005) noted that when controlling *S*. Dublin infection in cattle, persistently infected asymptomatic carriers are a problem for the spread of infection. Within adult cattle recovering from clinical salmonellosis, *S.* Dublin can persist within the lymph nodes and internal

organs resulting in periodic or intermittent excretion for up to several years without symptoms (Hansen *et al.*, 2005; Mateus *et al.*, 2008; Frost *et al*., 1997). As asymptomatic carriers can excrete bacteria in milk and faeces, the herd environment is contaminated which, if not effectively controlled for, can result in persistent intra-herd infection with the potential to spread inter-herd, to wildlife, farm hands and the public (Hansen *et al.*, 2005). Potentially, the prevalence of *S.* Dublin is underestimated due to it remaining latent within herds with a lack of clinical signs (Wray and Davies, 2000). As abortion may be the only clinical sign observed in asymptomatic pregnant cattle, *S*. Dublin should be considered amongst the causal agents during abortion investigations (Mateus *et al.*, 2008). The possibility for salmonellae to subsist amongst a seemingly healthy herd presents an infection control issue, posing a threat to cattle welfare with the potential of cattle failing to thrive.

There are three types of subclinical carrier; passive, latent and active (Nielsen, 2013). Passive carriers have *S.* Dublin within the lumen of the gut, no invasion of the intestinal epithelium, and shed the bacteria periodically in faeces for weeks to months. Latent carriers have *S*. Dublin persist within lymphoid tissues, for months to years, however they do not shed unless reactivated. Reactivation may occur from stress or other unknown causes, upon which low amounts of bacteria are shed (Nielsen, 2013). Active carriers can carry *S.* Dublin within the lumen of the gut, gut-associated tissues, the lymphoid system and internal organs, resulting in either intermittent or continuous shedding at large amounts (Nielsen, 2013).

Dependent on infection, heifers and cows had significantly greater probability of becoming carriers around the time of calving than cows in mid to late lactation (Nielsen *et al.*, 2004). This suggests that stress is an important risk factor in the pathogenesis of carrier development. Additionally, the stress of calving can often induce intermittent shedders to begin shedding *Salmonella* sp. (Nielsen *et al.*, 2004; Nielsen, 2013). As milk acts a vector for S. Dublin, this puts susceptible calves at an increased risk (Nielsen, 2013). The tendency of *S.* Dublin to produce long term carriers that periodically shed bacteria into the environment, contributing to the spread of infection, creates a major issue for control

of *S*. Dublin infections in cattle herds and perpetuates epidemiological factors. A rapid and inexpensive diagnostic kit would be a useful in this situation ensuring *Salmonella* infections are controlled. Additionally, cost effective tools would aid in the surveillance and control of *Salmonella* in cattle (Moore and Feist, 2006; Nielsen, 2012; Jadidi *et al.*, 2012; Nyman *et al.*, 2013). Nielsen (2012) suggest that a cost-effective method would enable studies to include larger samples of cattle within the research area, strengthening results.

It is important to have effective, cost efficient and reliable diagnostic tools for the detection of persistently infected animals to help control the spread of infection within and between cattle herds efficiently (Lomborg *et al.*, 2007). It is also important to correctly identify the causal agent during outbreaks of *Salmonella* in cattle (Baggesen *et al.*, 2006). To achieve correct identification, Lomborg *et al.* (2007) notes the requirement for tests with high predictive values to enable large scale screenings. *Salmonella* sp. have a high impact on economics and animal welfare, with an increased risk to calves.

1.4.1 Aetiological agents of calf scour and antibiotic stewardship

Diarrhoea is the most common symptom of salmonellosis in cattle; however, *Salmonella* sp. is not the only aetiological agent of scour. Viruses, such as Bovine Viral Diarrhoea, and parasites, such as lung worm, can also be causal agents of diarrhoea in cattle. Salmonellosis can kill calves within 48 hours, which with current methods is quicker than a diagnosis (Nielsen, 2013). Thus, when presented with newly born calves suffering from scour, prophylactic treatment with antibiotics to stave off potential salmonellosis is common, despite a variety of potential causal agents. With the increase in antimicrobial resistance, this is a cause for concern.

Antimicrobial resistance, when microorganisms change in ways that render current antimicrobial treatments useless, is a global phenomenon. The World Health Organisation (WHO) has called for a global concerted effort to slow the development of resistance. WHO focus on several aspects

including; antimicrobial stewardship to conserve the effectiveness of existing treatments, and encouraging the development of new antibiotics, diagnostics and novel therapies (Shallcross and Davies, 2014). WHO noted *Salmonella* sp. as 'high priority' in terms of developing new antimicrobials (Tacconelli *et al*., 2017).

Multidrug resistance (MDR) within *Salmonella* sp. can be defined as an isolate which is non-susceptible to at least one agent out of at least three antimicrobial agent classes (Magiorakos *et al.*, 2011). The emergence of MDR *Salmonella* strains is beginning to limit treatment options within cattle herds (Costa *et al.*, 2012). Mastroeni *et al.* (2000) noted that multi-drug resistant *Salmonella* strains are emerging and that the efficacy of currently available *Salmonella* vaccines is sub-optimal. Therefore, to ensure that the impact of salmonellae upon health and welfare is reduced, quick and reliable diagnostics that allow for targeted treatment are required (Mastroeni *et al*., 2000). *S. enterica* has mechanisms to resist antimicrobial peptides produced by the host in order to survive and colonise the gastrointestinal tract, causing disease (Elfenbein *et al.*, 2013; Kim, 2003). The emergence of antimicrobial resistance (AMR) is a prominent concern, *Salmonella* sp. are adapted to invade the gut, with AMR salmonellosis will become harder to treat, resulting in increased morbidity and mortality (Filioussis et al., 2008).

A study by Adhikari *et al.* (2009) monitored the appearance of MDR strains in cattle in Washington State, America. Among the historic clinical MDR strains, *S.* Typhimurium was the most common serovar (12/26) followed by *S*. Newport (9/26). Among 13 herds positive for a history of clinical salmonellosis before the study, 5 farms were positive for MDR *Salmonella* upon the first visit and 8 acquired new MDR strains on the second visit or later. Adhikari *et al.,* (2009) observed a total of 70 new MDR *Salmonella* strain introductions in 33 herds. This study shows the potential for salmonellae to transfer inter-herd, with MDR *Salmonella* strains there is an increased difficulty in eradicating the disease fully from a herd. Multiple antibiotic courses, isolation of infected animals and cattle mortality result in increased costs, with salmonellosis reducing the health and welfare of the livestock. To avoid

the knock-on effects of MDR salmonellae, treatment needs to be targeted. The detection and identification of bacterial pathogens from clinical samples is crucial to determine the cause of infection and to direct antimicrobial therapy, which should help reduce the proliferation of MDR strains whilst improving outcomes and decreasing costs (Francois *et al.*, 2011).

Current antimicrobials need to be safeguarded and the spread of MDR strains needs to be controlled, targeted treatment is needed to confirm that antimicrobials are only administered in the presence of a bacterial infection. To ensure this, quicker methods of *Salmonella* sp. detection is needed to determine the cause of scour in ailing calves.

1.5 Rapid Diagnostics to enable targeted treatment of Salmonellosis

The development of robust and rapid diagnostic tests are needed to enable point of care detection and targeted treatment to improve welfare, limit loss of product and help control MDR strains, as well as safeguarding public health and controlling the spread of infection.

For effective diagnosis of infection with *Salmonella* sp., stool culture is considered the gold standard method for the microbiological identification of the organism (Falkenhorst *et al.*, 2013; Nielsen, 2013). Faecal samples are inoculated onto an enrichment medium, further cultivated onto a selective medium, then biochemical or molecular analysis is used to confirm *Salmonella* sp. presence and to determine the serotype, often taking a few days for a negative result and longer to confirm presumptive isolates (Vo *et al.*, 2006; Falkenhorst *et al.*, 2013; Nielsen, 2013). However, bacterial culture is time consuming, has relatively low sensitivity and is laborious (Mateus *et al.*, 2008; Jadidi *et al.*, 2012; Nielsen, 2012; Falkenhorst *et al.*, 2013; Nyman *et al.*, 2013). Several factors, including competing flora, can affect the culturing method which can result in differing outcomes when isolating *S.* Dublin (Baggesen *et al.*, 2006). Additionally, due to intermittent shedding and differing infection symptoms of *S.* Dublin and other *Salmonella* sp. within cattle, detection of *S.* Dublin from faecal

culturing can be problematic (Baggesen *et al.*, 2006). The sensitivity for stool culture is poor, 16-20% when used to diagnose carrier animals with intermittent shedding (Nielsen, 2013). However, when used in conjunction with a rapid detection method, culturing is useful to determine the serotype of the strain; when a strain is persistent within a herd, when an animal is acutely ill, or for research purposes (Nielsen, 2013).

1.5.1 Rapid diagnostics: nucleic acid amplification

Due to the widespread challenges presented by *Salmonella* infection, the need for the development of rapid and sensitive methods for diagnosis is clear.

Nucleic acid amplification is a valuable tool in the diagnosis of infectious diseases, and, amongst various nucleic acid amplification methods, polymerase chain reaction (PCR) is the most widely used (Nagamine *et al.*, 2002; Parida *et al.,* 2008). PCR is one of the most sensitive diagnostic methods; it is quicker and more specific than culturing. However, Parida *et al.* (2008) cautions that PCR-based methods require expensive high precision instruments or elaborate methods for detection of the amplified products. Extensive sample preparation is often required to eliminate contaminates that interfere with PCR amplification: the protocols can be cumbersome to adapt and are often labour intensive, specialised operators are required (Notomi *et al.*, 2000; Francois *et al.*, 2011; Parida *et al.*, 2008; Fredricks and Relman, 1998). The process is time-consuming; post-PCR target identification methods, such as gel electrophoresis, are often required. To ensure targeted treatment, to reduce MDR salmonellae and improve cattle morbidity, faster, simpler detection methods are needed.

However, methods to increase the speed of PCR and reduce the laborious nature of the procedure have been developed. Pathmanathan *et al.* (2003) developed a PCR protocol that can detect *Salmonella* sp. in under 18hrs by using human faecal samples directly without extracting genomic DNA cutting out the need for a lengthy DNA extraction procedure. Whilst they noted that the sensitivity was reduced by normal flora and inhibitors in the stool sample, they utilised a 10- and 20-fold dilution

method which reduced the interference. Madi et al. (2012) created a real-time reverse transcription - PCR (rRT-PCR) protocol that can run to completion in 60mins, using an Enigma FL thermocycler, to detect foot and mouth disease in cattle. They note that the study is an important step in the deployment of rRT-PCR tests for field diagnosis, but further studies are needed to develop commercially viable consumables as the protocol uses 'wet' reagents. When compared to conventional PCR methods these techniques are simpler whilst retaining their sensitivity and specificity. However, both methods currently require expensive laboratory-based equipment and specialised operators.

Loop mediated isothermal amplification (LAMP) works at a constant temperature using a strand displacement reaction (Notomi *et al.*, 2000). Nagamine *et al.* (2002) showed that the inclusion of loop primers in LAMP enables DNA amplification in less than 30 minutes, as well as providing higher sensitivity. The method is simple, reliable and rapid (Notomi *et al.*, 2000; Parida *et al.*, 2008). Okamura *et al.* (2008) noted that the advantages of this include the prevention of contamination and removal of the need for complicated temperature control. Additionally, Parida *et al.* (2008) observed that LAMP shows a high specificity and high amplification efficiency and would be suited to clinical diagnosis. LAMP assays are completed in a single reaction tube, reducing the risk of contamination and enabling field use due to cheap consumables. Visualisation of results, without the need for post-amplification electrophoresis, can be achieved relatively easily either through observing the turbidity or a colour change from a fluorescent intercalating dye (Parida *et al.*, 2008).

LAMP is robust nucleic amplification technique; LAMP showed a superior tolerance to biological substances and sub-optimal assay conditions over PCR (Kaneko *et al.*, 2007; Yang *et al.*, 2013). These findings were supported by Francois *et al.* (2011), in a study that tested the robustness of LAMP assays detecting *Salmonella enterica* serovar Typhi (*S.* Typhi) for diagnostic use in developing countries. Francois *et al.* (2011) used various LAMP kits to detect *S.* Typhi in human stool and urine samples. The

authors showed the stability of LAMP assays, which yielded reproducible results through a broad range of temperatures, elongation times and pH values, and robustness despite the presence of untreated urine and stool samples. Francois *et al.* (2011) suggest that LAMP is a useful option for rapid detection; however, the multiplexing ability has yet to be demonstrated. In conclusion, Francois *et al.* (2011) noted that LAMP is not only sensitive, but fast, and highly robust under circumstances of impure preparations and variable incubation times. In comparison they found that it would be unlikely that PCR could be adapted for widespread deployment in the developing world due to the cost, specialised equipment needs and stringent technical requirements of the method.

To reduce the burden of bovine salmonellosis effectively and cost efficiently, rapid and sensitive diagnostics should be used for the detection of *Salmonella* sp. to control the spread of infection (Wattiau *et al*., 2011; Lomborg *et al.*, 2007). New methodologies for the identification of *Salmonella* should be rapid, robust, reliable, portable, and sensitive, producing objective results (Wattiau *et al,* 2011; Lomborg et al., 2007). A LAMP assay that is pan-*Salmonella* sp. would meet these requirements, with the potential to be used at the point of decision making by adapting the method for naked-eye results.

1.5.2 Rapid diagnostics: Immunoassays and biosensors

Within clinical diagnosis, immunoassays have been particularly effective, with Enzyme-Linked Immuno-Sorbent Assays (ELISA) becoming the gold-standard (Zhu *et al.*, 2019; Mobed *et al.,* 2019; Holford *et al*., 2012). However, immunoassays can be time consuming, labour intensive, and expensive: ELISA requires several working, incubation and washing steps that do not allow for immediate treatment (Ewald *et al*., 2013; Holford *et al.*, 2012). By combining the sensitivity and specificity of immunoassays with biosensors, the issues commonly associated with immunoassays could be solved (Holford *et al*., 2012).

Nyman *et al.* (2013) evaluated and compared three ELISA that used bulk milk samples to diagnose *Salmonella* sp. using a protocol that could be completed in less than two hours. Overall, they found high specificity using the ELISA method. The method is fast and relatively simple, however due to the speed with which *Salmonella* sp. can result in mortality for calves, a readable immune response is unlikely to be mounted in time for an ELISA to recognise Salmonellosis in a calf (Nielsen, 2013). Nyman *et al.* (2013) concluded that ELISA was a good complement, but would never replace, bacteriology in *Salmonella* screening in Sweden. Hansen *et al.* (2005) developed an ELISA to differentiate between acute and persistent infection of *S.* Dublin in cattle to identify carriers. The use of ELISA may be better suited to academic research and screening methods, rather than larger scale rapid diagnostics within cattle.

Ewald *et al.* (2013) noted that, especially within large animal farms, costs per test are a matter of importance, advising that cheap, reliable and time-efficient methods, as well as portable devices, would allow for quick counter measures to avoid the spread of infection. Ewald *et al.* (2013) developed a biosensor platform allowing for label-free detection of diagnostic markers in animal serum based upon the principles of Reflectometric Interference Spectroscopy (RIfS). RIfS is a direct optical method, which cuts out the need for expensive labelling reagents. Ewald *et al.* (2013) suggest that the sensor platform presented is robust and portable with capability to be used with cheap consumables. The team developed a method that uses lipopolysaccharides to recognise *S*. Typhimurium, however as the preparation has two 24hr steps this technique is not as rapid in relation to other molecular methods.

Electrochemical biosensors are low cost, with high sensitivity, fast response, low sample volumes, and easy operation without the need for expensive instrumentation or specialised personnel, as well as the potential for mass fabrication (Konchi *et al*., 2007; Holford *et al*., 2012; Bahadir and Sezginturk, 2015; Kokkinos *et al*., 2016). Immunosensors are a type of electrochemical biosensor, detecting antibody-antigen interactions on a transducer surface linked to an electrode (Felix and Angnes, 2018).

A commercialised system, the Vantix Research tool (Vantix™ Ltd, Cambridge, UK), utilises a novel biosensor that can be used as a platform for an immunoassay which has been reported to be simple, practical, and cost-effective (Purvis *et al*., 2003; Stead *et al*., 2011; Cork *et al*., 2012). Without the need for specialist biosensor knowledge, the Vantix platform allows for the adaption of existing and established ELISA protocols achieving the same sensitivity and specificity as the parent ELISA but with greatly reduced protocol and run times (Purvis *et al*., 2003; Stead *et al*., 2011; Cork *et al*., 2012). Rapid, simple Vantix assays would enable quick and efficient turnaround either on farm or in diagnostic laboratories, allowing targeted treatment, improving cattle prognosis and reducing costs (Cork *et al.*, 2012).

Rapid diagnostics to detect *Salmonella* would enable targeted treatment of the disease by reducing diagnosis time, allowing a potential reduction of bovine salmonellosis and reducing AMR. This could lead to a reduction of contamination in the food supply and environment, increase the welfare of cattle, decrease economic impact of *Salmonella* on the cattle industry, improve public health and help safeguard the effectiveness of current antimicrobials (Elfenbein *et al.*, 2013).

The aim of this study is to develop a rapid, reliable, and robust method for pan-*Salmonella* detection through calf scour, that can be used on-site to allow for targeted treatment of ailing calves. To achieve this, the following objectives;

- To develop a loop-mediated amplification assay targeting *Salmonella* sp.
- To develop a potentiometric immunoassay targeting *Salmonella sp.* using the Vantix System
- To ensure that visualisation of positive *Salmonella* sp. detection is clear, definitive, and easy to interpret for both diagnostic methods
- To ensure that both diagnostics can detect multiple *Salmonella* serovars, specifically *S.* Dublin as it is host adapted to cattle, with high sensitivity and specificity

- To ensure that when calf scour is used as a test sample, both diagnostic assays able to detect *Salmonella* sp., despite the antagonistic sample
- To optimise both diagnostic methods to allow for rapid detection, without losing sensitivity

2. Methodology

2.1. Bacterial isolates, storage, and growth conditions

All isolates and reference strains were stored in cryovials (Microbank, Prolabs Diagnostics) at -80°C and were revived before use in further experiments by culture onto Brain Heart Infusion Agar (Oxoid, CM1136) aerobically overnight at 37°C. Cryovials were kept frozen when in use outside of the freezer. To revive isolates, aseptic technique was used to take a swab from the cryovial which was streaked onto a brain heart infusion agar plate.

2.1.1. Reference strains

Unless otherwise stated, lab strains *S.* Dublin (NCTC: 12710, PHE – Culture Collections) and *Salmonella enterica* serovar Mbandaka (*S.* Mbandaka, NCTC: 07892, PHE – Culture Collections) were used as positive controls, with *Escherichia coli* (*E.coli,* NCTC: 12241, PHE – Culture Collections) as a negative control.

2.1.2. Handling of cattle faecal samples

Faecal matter, provided by Westpoint Farm Vets, was tested for *Salmonella* sp. and separated on arrival into 1ml aliquots within sterile 2ml microcentrifuge tubes and stored at -80°C for long term storage. For use, faecal matter was thawed at 4°C, overnight and used within 24hrs.

2.1.2.1 Isolation of Salmonella sp. from cattle faecal samples

To isolate *Salmonella* sp. from faecal matter, a sample of faeces was placed in 10ml of peptone buffered water (Oxoid, BO0688) and incubated aerobically overnight at 37°C. The sample was then vortexed, swabbed into 10ml Rapport Vassiliadis broth (Oxoid, CM0866) and incubated aerobically overnight at 37°C. A loopful was then streaked onto Brilliant Green agar (BGA – Oxoid, CM0263) and Xylose-Lysine-Desoxycholate agar (XLD – Oxoid, CM0469) and incubated aerobically overnight at 37°C.

All red-pink-white opaque colonies on red BGA, and black colonies on red XLD, with differing morphology within the plate (differences in size, shape, elevation, texture), were then purity streaked

onto Nutrient agar (NA – Oxoid, CM0309) and incubated aerobically overnight at 37°C. Biochemical confirmation to genus level was completed using an API 20E (bioMérieux) strip which was incubated for 18hrs at 37°C. (Method modified from Public Health England (PHE), 2014).

2.1.3. Isolates used for PCR assays

Isolates used to extract genomic DNA to enable PCR assay included NCTC *Escherichia coli* (*E. coli* – NCTC: 12241) which was used as a negative control, with 7 *Salmonella* clinical isolates, provided by Dr. Rob Davies at Animal and Plant Health Agency (APHA), Weybridge, as test strains. These strains were isolated from cats and dogs. The NCTC *Salmonella* strains were not used within PCR experiments.

2.1.4. Isolates used for LAMP and immunoassays

Positive and negative controls were as in 2.1.1. Known field strains, provided by Dr. Phil Wakely at APHA, Weybridge, were used. The isolates were confirmed to genus level as *Salmonella* sp. Strains included; *Salmonella enterica* serovar Agama, *S.* Dublin, *Salmonella enterica* serovar Mbandaka (*S.* Mbandaka), *Salmonella enterica* serovar Montevideo (*S.* Montevideo), *S*. Typhimurium (strain: DT104) and *Salmonella enterica* serovar Newport (*S.* Newport). These clinical strains were isolated from cattle, and were procured in Dec 2015 from APHA. Deemed more relevant to this study than the strains provided by Dr. Rob Davies in section 2.1.3, these strains were used in all LAMP and immunoassays.

2.2 Bioinformatics methods

2.2.1. Genomes used within Mauve genome alignment.

Salmonella genomes were collected from the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/genome/microbes/) for genomic alignment to detect conserved sequences across multiple *Salmonella* genomes. Only complete genome sequences were used, from those available at the time genome selection occurred, Jan 2015 - see table 2.1.

Table 2.1: Complete genomes used in Mauve to determine highly conserved pan-*Salmonella* sp. genes.

Salmonella serovar	Strain I.D	GenBank accession No.
Choleraesuis	SC-B67	NC_006905
Dublin	CT_02021853	NC_011205
Enteritidis	EC20121176	CP007270
Enteritidis	P125109	NC_011294
Gallinarum (Pullorum)	RKS5078	Nc_011274
Gallinarum	287,91	NC_016831
Newport	SL254	NC_011080
Newport	USMARC-S31241	NC_021902
Typhi	CT18	NC_003198
Typhi	TY2	NC_004631
Typhimurium	14028S	NC_016856
Typhimurium	LT2	NC_003197

2.2.2. Genome alignment using Mauve

Genomes (see Table 2.1) were aligned using Mauve (Version 2.4.0), multiple genome alignment software, which allows for research into genome-wide evolutionary dynamics and comparative genomics (Darling *et al.*, 2004). Sequences were entered in a Genbank format to allow for visualisation of annotated genes. A full alignment employing ProgressiveMauve was used with parameters set for an alignment of closely related genomes (Darling *et al*., 2010).

Within ProgressiveMauve, a full alignment with iterative refinement was used; the most in-depth alignment, using MUSCLE to generate a recursive anchor and then to refine the alignment (Darling *et al*., 2010). Largely default parameters were used within the alignment as ProgressiveMauve defaults

for aligning closely related genomes with moderate to high amounts of rearrangement. Default seed weight was used, the program selects this based on the base length of sequences. When aligning highly divergent sequences this can be too conservative, however higher seed weights can reduce noisy matching (Darling *et al*., 2010). Collinear genomes were assumed and 'sum-of-pairs LCB scoring' was disabled, due to *Salmonella* sp. being closely related.

Once aligned, the sequences were screened manually for highly conserved areas of sequence; see section 3.2.1 for a detail description of Mauve alignment navigation.

2.2.3. Specificity testing of genes and primers

To determine the specificity of gene targets gathered from the Mauve Alignment and the efficacy of all nucleic amplification primer sets generated in this study, the following method and definition were used unless otherwise specified.

To confirm specificity nucleotide Basic Local Alignment Search Tool (BLASTn; Altschul *et al.*, 1990; Altschul *et al.*, 1997) was used to screen for unintentional and non-specific sequence matches. Sequences were submitted in FASTA format, unless otherwise stated.

An unintentional match was considered as a match within the *Salmonella* genome but outside of the targeted sequence. Unintentional matches and confirmation of pan-*Salmonella* specific sequences were investigated by using a BLASTn search that was filtered within 'search set' as 'organism = salmonella (taxid: 590)'.

A non-specific match was considered as a match not within the *Salmonella* genome. A BLASTn search was used with 'search set' filtered by 'organism = salmonella (taxid: 590)' with the 'EXCLUDE' option selected.

Matches were assessed for relevance in a cattle industry setting through literature research.

2.2.3.1 Definitions of conservation and specificity for genes

Genes gathered from the Mauve alignment were verified as highly conserved by noting how many serovars the conserved gene sequence occurred in and *Salmonella* sp. specific by screening for non-specific matches.

2.2.4 Primer generation for nucleic amplification techniques

The genes identified as pan-*Salmonella* specific & highly conserved using bioinformatics were targeted for all nucleic amplification primer generation. The genes targeted were *bapA*, *hilA* and *orgA*. Due to the *bapA* gene having a homologous sequence to *Citrobacter* sp. past 11,474 base pairs (bp) only the first 10,000 bp of the *Salmonella bapA* gene were considered for primer generation.

2.2.4.1. PCR primer generation using Primer BLAST

Primers for PCR testing were generated using primer BLAST (Ye *et al*., 2012). Unless otherwise specified default parameters were used. Once generated, primer sets were sent for synthesis (section 2.3.3) and used downstream within PCR assays, see table 2.4.

2.2.4.2. LAMP primer generation using PrimerExplorer V.4

For continuity, where able, the same gene targets were used for all nucleic amplification techniques. However, as LAMP primer sets require 6 primers per set, compared to two primers per set as in PCR the PCR primer set was redesigned for use in LAMP assays (Section 5.1 – comprehensive explanation of LAMP primers). Primers for LAMP testing were generated using PrimerExplorer V.4 (https://primerexplorer.jp/e/) and the guidelines provided alongside the software.

As PrimerExplorer V.4 only accepts sequence inputs of 2,000bp, the sections of the genes containing the PCR primer sequences, previously generated in Primer BLAST, were targeted. As *hilA* and *orgA* were relatively small genes (1662bp and 600bp respectively) the whole gene sequence was included

(Table 2.2). The *bapA* gene is larger than 2,000bp (11,474bp) therefore 2,000 bp of the gene sequence was selected with the PCR primer sequence used located in the middle of the section (Table 2.2).

Table 2.2. The positions of the PCR primers on the gene sequences used to generate LAMP primers.

Gene	Gene length (bp)	PCR Primer I.D	Nucleotide position of first PCR primer base on gene (bp)	2,000 bp sequence used to generate LAMP primers (bp)
bapA	11474	bapA_1	5100	4120 - 6120
		bapA_2	4080	3100 - 5100
hilA	1662	hilA_1	988	Whole gene
		hilA_2	664	Whole gene
orgA	600	orgA_1	138	Whole gene
		orgA_2	80	Whole gene

In general, LAMP primers were made as per the specifications within 3.2.3.

Once assessed for specificity as in section 2.2.4, primer sets were used to generate loop primers and the overall stability of the complete primer set was assessed. Where applicable LAMP primer sets containing the sequence targeted by the PCR primer sets were preferably chosen. For a detailed explanation of PrimerExplorer V4. see section 3.2.3. Once generated, primer sets were synthesised (section 2.3.3) and used downstream within LAMP assays (Table 2.5).

2.2.4.3 Specificity testing of primers

Primer sets were assessed as per section 2.2.3, as well as assessed for genome positioning upon an unintentional match: BLASTn was used to determine whether the match would generate a product. Primers positioned ≥10,000bp apart, or antisense from each other, were considered unlikely to generate a product.

2.2.4.3.1 Definitions of specificity for PCR primers

When assessed for non-specific matches, a non-specific match within a PCR primer set was considered as a match within/near the sequence targeted by the primers.

2.2.4.3.2 Definitions of specificity for LAMP primers

When assessed for unspecific matches, an unspecific match within a LAMP primer set was considered as a match between any forward primer and any backward primer.

2.3 Nucleic amplification for detection of *Salmonella* sp.

2.3.1. Isolation of genomic DNA for use in nucleic amplification

To prepare for DNA extraction, bacteria were inoculated into Brain Heart Infusion broth (BHI broth – Oxoid, CM1135) and cultured overnight at 37°C in a shaking incubator, after revival. Absorption (OD) were measured at 600nm on a spectrophotometer, with an aim of obtaining 1.0 OD units. Spectrophotometer was zeroed against BHI broth.

DNA extraction was completed using the GeneJet Genomic DNA Purification Kit (Thermoscientific, K0721) as per the Gram-negative bacteria genomic DNA purification protocol provided with the kit. Bacterial cells were harvested in a 2ml microcentrifuge tube by centrifugation for 10min at 5000xg and the supernatant was discarded. The pellet was resuspended in 180µl of digestion solution and 20µl of Proteinase K solution was added. Suspensions were vortexed and incubated at 56°C in a shaking incubator for 30mins. Following addition of 20µl of RNase A solution, samples were vortexed and incubated at room temperature for 10mins. To the sample, 200µl of Lysis solution was added and vortexed well, then 400µl of 50% ethanol was mixed in. The prepared lysate was then added to a DNA purification column within a collection tube. Columns were centrifuged for 1 min at 6000xg and collection tubes with flow-through solution were discarded. Columns were placed with a new collection tube and 500µl of wash buffer I was added. After centrifugation for 1 min at 8000xg the flow-through was discarded and 500µl of Wash Buffer II was added to the column. This was centrifuged for 3 mins at maximum speed (14,000xg) and the collection tube including the flow-through was discarded. Column were placed in a sterile 1.5µl microcentrifuge tube and 200µl of

elution buffer was added. After incubation at room temperature for 2mins and centrifugation for 1min at 8000xg, the purification column was discarded and, following quantification and quality checks on the NanoVue, as per the purification parameters in table 2.3., the purified DNA was stored at -20°C.

Table 2.3: The wavelengths and ratios used to ensure the purity of genomic DNA as well as the potential contaminates if absorbance is outside of the acceptable parameters (adapted from the Nanovue manual).

Wavelength (nm)	Acceptable purity parameters (Absorbance)	Absorbance outside of parameters	Potential contaminate
260/280	1.7-1.9	Deviations indicate presence of impurity in the sample	Protein presence
260/230	≥2.0	Lower than this could indicate impurities	Protein presence and potential buffer interference
260	≥0.1	Indicates inaccurate ratio measurements	-
320	≤0.1	Indicates background absorbance	Turbidity, stray particulates, or high absorbance buffer solutions

2.3.2. Gel electrophoresis for visualisation of nucleic amplification products

For all nucleic acid amplifications, the end-point results were visualised using gel electrophoresis. To allow visualisation of small nucleic product, 2% agarose gels were made by heating 1.5g of agarose with 75ml of 1x TBE buffer (section 2.3.2.1). Once cooled slightly, 9µl of SYBR safe (Invitrogen, S33102) was added to the agarose and using a cast and well-moulds, the gel was poured and set for approximately 30mins at room temperature. Once set, casts were submerged in 1x TBE buffer within a gel electrophoresis rig, well-moulds were then removed. Within the first well of each well row, GeneRuler 100bp DNA ladder (Thermoscientific, SM0241) was added, this ladder provides a range of 100bp-1,000bp. To nucleic amplification products, loading buffer (included with GeneRuler 100bp DNA ladder: Thermoscientific, SM0241) was added at 1:5 buffer to product ratio before being loaded into the wells of the gel. Gel Images were captured using Genesnap on a Syngene G-box.

2.3.2.1 TBE (Tris/Borate/EDTA) buffer

TBE buffer was prepared at 10x concentration by adding 0.89M Tris Base, 0.89M Boric Acid and 0.02M EDTA disodium salt to 500ml of distilled water. The pH was then adjusted to 8.0 and water was added to make a final volume of 1L. To get a working concentration of TBE, a 1:10 dilution was completed.

2.3.3. Oligonucleotide synthesis and storage for use in nucleic amplification assays

DNA Oligonucleotides were synthesised by Sigma Aldrich (in water, desalted). Upon delivery, lyophilised oligonucleotides were re-suspended in RNA-free water as per the protocol provided by Sigma and stored at -20°C in aliquots. Biswas *et al.* (2010) determined that their PCR primers targeting the *bapA* gene (Biswas_F and Biswas_B) were pan-*Salmonella* specific and were thus synthesised for use as a positive control within PCR assays. Yang *et al.* (2016) determined that Sal4 LAMP primer set was pan-Salmonellae specific and thus was synthesised for use as a positive control within LAMP assays.

Table 2.4: Oligonucleotides used within polymerase chain reactions, derived as per section 2.2.4.1.

Target	Lab Reference	Sequence (5'-3')	Product Length (bp)
bapA	BapA_1FP	CGGTGAATTCGTCGTTACGC	425
	BapA_1BP	GATCGACAGTGATCCCGACC	
	BapA_2FP	ATCGGCAATAATGGCGCAAC	591
	BapA_2BP	GATTTCATTGACGACGGGCG	
	Biswas_F	GCCATGGTGCTGGAAGGCCTGGCGGTT	667
	Biswas_B	GGTCGACGGGAAGGGTAAAATGACCTTC	
hilA	HilA_1FP	CGACAGAGCTGGACCACAAT	660
	HilA_1BP	TCAAGCGGGGATCCTGTTTC	
	HilA_2FP	ACCAACCCGCTTCTCTCTTG	344
	HilA_2BP	ATTGTGGTCCAGCTCTGTCG	
orgA	OrgA_1FP	GCGGCGGCAAATGAGTTAAT	384
	OrgA_1BP	AGCATCCTGCTTCAATGCCT	
	OrgA_2FP	TATCCATCCTCAGCGGTTGC	437
	OrgA_2BP	CCTGCTTCAATGCCTCCTCA	

Table 2.5: Oligonucleotides used within loop mediated isothermal amplification assays.

Gene Target	Primer set lab reference	Primer lab reference	Sequence (5'-3')
bapA	bapA1.1	bapA1.1_F3	CTCAACGGAACGGGAGAAG
		bapA1.1_FIP	CGCTTTGATCTACCGTGGCGCGCCACGATCCGCATTC
		bapA1.1_FLoop	AACCGATTTCTACGCC
		bapA1.1_BLoop	GCCGTAGCGACCGAT
		bapA1.1_BIP	GAGAGCAACGCGCACATCTGCGTAAAGCCGTCCGAAGG
		bapA1.1_B3	GTGATAACCGGCACATCTGG
	bapA1.2	bapA1.2_F3	AGTCCAGACGGTGGATGAC
		bapA1.2_FIP	CCAGGGTGCCATCGATATGATGGCGCGTCGCCGGAATT
		bapA1.2_FLoop	ACGGTAGCGTAAGGGTCG
		bapA1.2_BLoop	GCAAACCGATGGCGGTAC
		bapA1.2_BIP	GTCGTTACGCTCAGTCCGGCGCGCGATCGATAGCAAT
		bapA1.2_B3	CGTAGCCGGGCCGTTAT
	bapA2.1	bapA2.1_F3	CCGGCACCATCATCACC
		bapA2.1_FIP	AACCCTTCGCTCAGATTACGGGACTGGCTACCGTCCAGGTC
		bapA2.1_FLoop	TAGCGGATAGGTCCAGCTACC
		bapA2.1_BLoop	CCGACCTCCGGCGTTTT
		bapA2.1_BIP	ACGGATGCCGCAGGCAAGGCTGGGTATCAAGGGTAAC
		bapA2.1_B3	TTAGCGGCGCGTCAGG
	bapA2.2	bapA2.2_F3	CCCTGACTGCCATTGCC
		bapA2.2_FIP	GAACGGTGTCGACGGTGAAGGGATGCCGCCGGAAACAG
		bapA2.2_FLoop	GCTGTTCGATACGCCGCTG
		bapA2.2_BLoop	TAACCGATGGCGCCTTTACTAACG
		bapA2.2_BIP	TTGCACCAGTGACCGGGCTTCGCCGCTGCCGTTAA
		bapA2.2_B3	CGCCATTGTCGTAAATCGTG
hilA	hilA1	hilA1_F3	CGCTCAGAAAAGAAAGTCAAT
		hilA1_FIP	TCCAGTAAGGTGTTTTTACTCACAAATTCCGCCAAAAGAATATGC
		hilA1_FLoop	GCAGGATGACCAGAACG
		hilA1_BLoop	TCTCTTACCCGCTGT
		hilA1_BIP	CGACGCGGAAGTTAACGAAGAGAATACGTCGTAAGGCAT
		hilA1_B3	TGTTTCAATGTAACGATGCT
	hilA2	hilA2_F3	CTACGCTCAGAAAAGAAAGTC
		hilA2_FIP	AAGGTGTTTTTACTCACAATCTCGCAATATTCCGCCAAAAGAATATGC
		hilA2_FLoop	CAGGATGACCAGAACG
		hilA2_BLoop	TCTCTTACCCGCTGT
		hilA2_BIP	GCGACGCGGAAGTTAACGAAAGAATACGTCGTAAGGCAT
		hilA2_B3	TGTTTCAATGTAACGATGCT
orgA	orgA1	orgA1_F3	TCCTCAGCGGTTGCAGAT
		orgA1_FIP	CTCCGTTCTTAAGCCGCCATGCGCGCCGGAAATGATTGTCA
		orgA1_FLoop	CGCCAGTATTAACTCATTTGC
		orgA1_BLoop	GTCAGTGGCGCCGACT
		orgA1_BIP	CTCACTGACGCAGCTGTGGCTGGCAACCGAGTAAATACGC
		orgA1_B3	TGCCAGATCGGCTCTCAG
	orgA2	orgA2_F3	TCCTCAGCGGTTGCAGAT
		orgA2_FIP	CTCCGTTCTTAAGCCGCCATGCGCGCCGGAAATGATTGTCA
		orgA2_FLoop	CGCCAGTATTAACTCATTTGC
		orgA2_BLoop	GCCGACTGCCGCAAGT
		orgA2_BIP	CTCACTGACGCAGCTGTGGCTGGCAACCGAGTAAATACGC
		orgA2_B3	CTTGCCAGATCGGCTCTC
invA	Sal4	Sal4_F3	GAACGTGTCGCGGAAGTC
		Sal4_FIP	GCGCGGCATCCGCATCAATATCTGGATGGTATGCCCGG
		Sal4_FLoop	TCAAATCGGCATCAATACTCATCTG
		Sal4_BLoop	AAAGGGAAAGCCAGCTTTACG
		Sal4_BIP	GCGAACGGCGAAGCGTACTGTCGCACCGTCAAAGGAAC
		Sal4_B3	CGGCAATAGCGTCACCTT

2.4. Polymerase Chain Reaction for the detection of *Salmonella* sp.

Gel electrophoresis was used to visualise all PCR results, see 2.2.2. Unless otherwise stated template DNA was at a concentration of 100ng in the overall reaction volume and primers were at a concentration of 2µM in the overall reaction volume.

2.4.1. PCR assays using the Dream *Taq* Green master mix

To determine whether highly conserved genes selected from the Mauve genomic alignment can detect multiple *Salmonella* strains, PCR assays were completed using the PCR primers developed using Primer BLAST (see table 2.4) and Dream *Taq* Green PCR Master Mix kit (Dream *Taq* – Thermoscientific, K1081). The method was adapted from the protocol provided with the Dream *Taq* Green Master Mix. Reagents were added as per table 2.6, to a 0.2ml microcentrifuge tube, and added to a thermal cycler set with the cycling parameters in table 2.7 to enable amplification. The end concentration within the final reaction volume of primers was 2µM and for DNA was 0.1µg.

Table 2.6: Reagents used for PCR assays using the Dream *Taq* Green Master mix

Reagent	Volume (µl)
Dream *Taq*	25
Forward primer	2
Backwards primer	2
Template DNA	1
Nuclease free water	20
Total volume (µl)	**50**

Table 2.7: The optimised cycling conditions for the thermal cycler to enable amplification of DNA for PCR assays using Dream *Taq* Green Master Mix

Step	Temperature (°C)	Time (mins)	No. of Cycles
Initial Denaturation	95	1.5	1
Denaturation	95	0.5	30
Annealing	65	0.5	
Extension	72	0.5	
Final Extension	72	10	1

2.4.2. PCR assays using the HotStarTaq Plus Master mix

To determine whether highly conserved genes selected in the Mauve genomic alignment can detect multiple *Salmonella* strains, PCR assays were completed using the PCR primers developed using Primer BLAST (Table 2.4) and HotStarTaq Plus PCR Master Mix kit (HotStarTaq – Qiagen, 203643). Protocol was adapted from the protocol provided with the HotStarTaq Plus master mix, reagents were added to a 0.2ml microcentrifuge tube as per table 2.8. The end concentration within the final reaction

volume of primers was 2µM and for DNA was 0.1µg. Assay tubes were added to the thermal cycler and products were amplified as per the cycling conditions in table 2.9.

Table 2.8: Reagents used for PCR assays using the HotStarTaq Plus mastermix

Reagent	Volume (µl)
HotStarTaq	10
Forward primer	1
Backwards primer	1
Template DNA	0.55 – 3*
Nuclease free water	5 – 7.45*
Total volume (µl)	**20**

Table 2.9: The optimised cycling conditions for thermal cycling to enable amplification of DNA for PCR assays using HotStarTaq mastermix

Step	Temperature (°C)	Time (mins)	No. of Cycles
Initial Denaturation	95	5	1
Denaturation	94	0.5	30
Annealing	55	0.5	
Extension	72	1	
Final Extension	72	10	1

*volumes vary due to concentration differences in Template DNA. Concentration of template DNA in overall mix 100ng. Overall volume made up to 20µl with Nuclease free water.

2.5 Loop mediated isothermal amplification for the detection of *Salmonella* sp.

All LAMP products were visualised using gel electrophoresis, see 2.3.2, and each assay was completed in triplicate. Template DNA was standardised to 6,000 copies of genomic DNA, unless otherwise stated. For all LAMP assays the following controls were used;

- a 'no template DNA' control, where template DNA/sample was replaced with sterile water
- a positive control, using Sal4 LAMP primers (Yang *et al*., 2016) and *Salmonella* sp. DNA
- a negative control, using test LAMP primers and *E.coli* (NCTC: 38173) DNA

The minimum amount of DNA LAMP assays are reported to detect is 6 copies of genomic DNA (Notomi *et al*., 2000). Unless otherwise stated, 6,000 copies of genomic DNA was used. To determine the molecular weight of genomic *Salmonella* sp. DNA the following was done;

- Molecular weight of a single genome was determined, using *S.* Typhimurium (LT2) genome

Amount of A → T bonds = 1160904
Amount of T → A bonds = 1159903 } x 667.41 (molecular weight of bp) = 1548929800

+

Amount of G → C bonds = 1268221
Amount of C → G bonds = 1268422 } x 686.41 (molecular weight of bp) = 1741177122

Molecular weight of genome = 3290106922

Molecular weight of *S.* Typhimurium genome was divided by Avogadro's number ($6.023*10^{23}$ molecules/mole) then multiplied by the number of copies required (6) equalling the mass of 6 copies ($3.277543x10^{-14}$). Whilst developing the assay, a good level of amplification was required, thus a concentration of 10,000 genomic copies per reaction was used, therefore the mass was divided by the volume to give a concentration of 0.011ng/µl of genomic DNA per reaction.

2.5.1. LAMP assays using the Optigene protocol to detect *S.* Dublin

To determine whether the LAMP primers generated using PrimerExplorer V4., table 2.5, are specific, LAMP assays were completed using the protocol provided by Optigene (Horsham UK). As LAMP requires six primers per set, stock primer mixes were created for each set, as per table 2.10. In the final reaction volume, LAMP primers were in the following concentrations; 0.8µM each of FIP/BIP, 0.4µM each of FLoop/Bloop, 0.2µM each of F3/B3 (Nagmine *et al*., 2002). Primer ser bapA1.1 was used, with *S.* Dublin (NCTC: 12710) as the *Salmonella* template DNA, for which the final concentration within the reaction mixture was 0.01ng/µl.

Reagents were added to 0.2ml microcentrifuge tubes, as per table 2.11, and placed in a hotplate for an hour at 65°C. Reaction tubes were manually checked for turbidity, by comparison with the 'no template DNA' control, every 5 minutes. To terminate the assay, tubes were transferred to a hotplate at 85°C for 10mins to inactivate the DNA polymerase.

Table 2.10: The protocol for generating the stock primer mix used within table 2.11

Reagent	Vol (ul)
Sterile Water	86
F3	1
B3	1
FLoop	2
BLoop	2
FIP	4
BIP	4
Overall	**100**

Table 2.11: The reaction mix for LAMP assays using the Optigene method.

*for primer mix see table 2.11

Reagent	Volume (µl)
Turbidometric isothermal mastermix (Optigene, ISO-001t)	15
Primer mix*	5
Template DNA	5
Total volume (µl)	25

2.5.2 Different detection techniques for LAMP assays to allow for visual detection of *Salmonella* sp.

2.5.2.1. Visualisation of LAMP product via turbidity

To optimise the Optigene protocol, section 2.5.1, methodology from Mori *et al.* (2001) was adapted, see table 2.12, to allow for increased visual turbidity. Visual turbidity is defined at clouding of the assay mix when compared to the no template DNA control. To allow space for additional reagents without changing reaction volumes, the concentrations within the stock primer mix were doubled, as per table 2.13, concentrations within the final reaction remained as stated in section 2.5.1. Reagents were added to 0.2ml microcentrifuge tubes, as per table 2.12, and placed on a hotplate at 65°C for an hour. Microcentrifuge tubes were manually checked every 5 minutes and compared to the 'no template DNA' control to check for visual turbidity. The experiment was terminated for 5 mins at 85°C. Primer set bapA1.1 was tested using *S.* Dublin (NCTC: 12710) in all test samples.

Table 2.12: LAMP protocol optimised from table 2.10 to allow for the visual observation of turbidity

*for primer mix see table 2.13

Reagent	Volume (µl)
Turbidometric isothermal mastermix (Optigene, ISO-001t)	15
Primer mix*	2.5
$MgSO_4$ (2mM)	0.5
Betaine (0.8M)	4
Template DNA	3
Total volume (µl)	25

Table 2.13: The protocol used to generate the primer mix used within LAMP assays optimised for visual turbidity, see table 2.12. Concentration within the primer mix doubled to allow for smaller volumes within reaction tubes.

Reagent	Vol (ul)
Sterile Water	44
F3	4
B3	4
FLoop	8
BLoop	8
FIP	16
BIP	16
Overall	**100**

2.5.2.2. Colorimetric assays for LAMP product visualisation

To enable visualisation of the LAMP products several dyes that undergo a colour change when in the presence of high quantities of DNA/Mg^{2+} were used, with an adaptation to the Optigene protocol used in 2.5.1. Reagents were added to 0.2ml microcentrifuge tubes as seen table 2.14 and sterile water was used to maintain reaction volume. Reaction tubes were placed in a hotplate at 65°C for an hour and checked for a change in colour, when compared to the no template DNA control, every 5mins. The reaction was terminated at 85°C for 5mins. For dye concentrations see table 5.1.

Dyes tested: Propidium Iodide, SYBR Safe, Nile Blue A, Methylene Blue, and Hydroxy napthol blue.

Table 2.14: Optigene methodology adapted to enable addition of dye for visualisation

Reagent	Volume (µl)
Turbidometric isothermal mastermix (Optigene, ISO-001t)	15
Primer mix*	2.5
Template DNA	2
Sterile water	4.5
Dye	1
Total volume (µl)	25

*Primer mix as seen in table 2.13

2.5.2.3. Fluorescent assays for LAMP product visualisation

To enable visualisation of the LAMP products several dyes that emit fluorescence when intercalated with DNA were tested. Reagents were added to 0.2ml microcentrifuge tubes as seen table 2.14. Reaction tubes were placed in a hotplate at 65°C for an hour and checked for fluorescence under a UV lamp, when compared to the no template DNA control, every 5 mins. The reaction was terminated at 85°C for 5mins. For dye concentrations see table 5.1.

Dyes tested: Propidium Iodide, SYBR Safe and SYBR green

2.6. Antibodies used within immunoassays to detect *Salmonella* sp.

For use within the immunoassays of this study three anti-*Salmonella* antibodies were initially selected, a fourth was later acquired (Table 2.15). Antibodies were stored long term at -20°C, aliquots for use were stored at 4°C for 1 week.

Table 2.15: The antibodies used within the study, with lab references and relevant information

Antibody	Lab ref	Isotype	Raised in	Type	Supplier	Information on reactivity (summarised from Supplier product info)
Salmonella Antibody (5D12A)	BMM	IgG1	Mouse	Monoclonal	Bio-rad	Broad Reactivity antibody, clone 5D12A recognises the core antigen that bears the O antigen. Antibody recognises *Salmonella enterica* serogroups; A (*S*. Paratyphi A), B (*S*. Typhimurium), C1 (*S*. Choleraesuis), C2, (*S*. Newport), D (*S*. Enteriditis), E1 (*S*. Anatum) and E2 (*S*. Selandia). Does not cross-react with *E. coli* 055: B5, *E. coli* K12 or *Klebsiella pneumoniae.*
Salmonella Group Antigen Antibody: HRP	BRP	IgG	Rabbit	Polyclonal	Bio-rad	Antibody is polyvalent for Salmonella O and H antigens, is unabsorbed and may cross react with related Enterobacteriaceae.
Salmonella Polyclonal Antibody	TRP	IgG	Rabbit	Polyclonal	Thermofisher	Antibodies was raised against a mixture of *S*. Enteriditis, *S*. Typhimurium and *S*. Heidelburg and are polyvalent for all "O and H" *Salmonella* antigens.
Salmonella LPS Monoclonal Antibody (A99H)	A99H	IgG2a	Mouse	Monoclonal	Thermofisher	Antibody is specific for common LPS core of all Salmonellae O-serogroups tested; A, B, C1, C2, D, E1, E3, E4, F, G1, G2. Does not cross-react with *E. coli*, *Klebsiella*, *Citrobacter, Pseudomonas, Yersinia, Shigella, Proteus* or *Legionella.*

2.6.1 Conjugating antibodies using Lightning-Link to allow for use in immunoassays

Antibodies that were not pre-conjugated to horseradish peroxidase (HRP) were conjugated using the Lightning-link HRP conjugation kit (Innova Biosciences Ltd), following the protocol provided within the kit. For each 10µl of antibody to be labelled, 1µl of LL-modifier was added and gently mixed. This solution was then aliquoted into the Lyophilised Lightning-Link mix vial and resuspended gently by pipetting. Vials were left at room temperature for minimum of 3 hours. For every 10µl of antibody used, 1µl of LL-quencher reagent was added and left at room temperature. After 30mins, conjugated antibody was either used immediately or stored at 4°C.

2.6.2 Antibody dilution for use within immunoassays

Antibodies were diluted with carbonate bicarbonate buffer (section 2.7.1) as needed for use within immunoassays; see sections 2.8 -2.9.

2.6.3 Adsorption of antibodies against *E. coli*

To remove cross-reactive antibodies from stock solutions, pre-diluted antibodies were adsorbed against *E. coli*. *E. coli* was prepared as per section 2.8.1. To 100µl of prediluted antibody, 10µl of *E. coli* suspension was added, vortexed gently and incubated for 1hr at 37°C. Antibodies were then centrifuged at 5000rpm for 10mins and the supernatant was removed and transferred to a sterile microcentrifuge tube. Supernatant was then used as antibody stock where stated.

2.7 Buffers and substrates for use with immunoassays

2.7.1 Carbonate Bicarbonate buffer

Carbonate Bicarbonate buffer was made with 3.03g of Sodium Carbonate (Na_2CO_3) and 6g of Sodium Bicarbonate ($NaCO_3$) in sterile water and pH was adjusted to 9.6 before making up a final volume of 1L.

2.7.2 Blocking buffer

Blocking solution was made with sterilised PBS containing 0.05% (v/v) Tween 20 and 0.1% (w/v) skimmed milk powder containing casein (Marvel, Sainsburys UK).

2.7.3 3,3',5,5'-tetramethylbenzidine (TMB) substrate

TMB substrate was made using the Pierce TMB Substrate Kit (Thermofisher, 34021) which detects horseradish peroxidase activity yielding a blue colour that changes to yellow (A_{max} = 450nm) upon

addition of sulphuric acid to stop the reaction. Immediately before use, equal volumes of TMB solution (0.4g/l) and Peroxide solution (0.02v/v Hydrogen Peroxide in citric acid buffer) were mixed.

2.8. ELISA for the detection of *Salmonella* sp.

Each assay was completed in triplicate. For all ELISAs the following controls were used, unless otherwise stated;

- No bacteria control, where the bacterial sample was replaced with un-inoculated carbonate bicarbonate buffer
- No antibody control, where the antibody was replaced with PBS containing 0.05% Tween 20.
- A negative control, using *E. coli*

2.8.1 Preparation of cultures for ELISA

Cultures were grown aerobically in 100ml nutrient broth within conical flasks on a shaking incubator at 37°C for 16-18 hours. Within falcon tubes, 20ml of the cultures were centrifuged at 5000rpm for 20mins. Pellets were then washed 3 times in 10ml PBS at 5000prm for 20mins. Pellets were then re-suspended in 10ml carbonate bicarbonate buffer and a stock solution of 0.25 OD units (equivalent to 10^8 cells/ml) was prepared using a spectrophotometer.

2.8.2 Direct ELISA protocol to determine sandwich assay antibody pairings

Using a 96 well plate (Nuclon flat), 100ul aliquots of stock culture solutions were added and incubated overnight at 37°C. Plates were washed 3 times with 200ul PBS per well using a multichannel pipette, before inversion and gently tapping dry on absorbent paper. Non-specific sites were blocked using 100µl PBS containing 0.05% (v/v) Tween 20 and 0.1% (w/v) skimmed milk powder containing casein (Marvel) for 1 hour at 37°C. Excess blocking solution was removed, and plates were washed thrice with PBS containing 0.05% (v/v) Tween 20. Antibodies conjugated with horse radish peroxidase were

diluted 1:500 and 100µl were added to the appropriate wells. Plates were then incubated for 2hrs at 37°C. Plates were washed thrice with PBS containing 0.05% (v/v) Tween 20 and 100µl of TMB substrate solution (section 2.7.3) was added to each well. Plates were developed at 10 minutes and the reaction was stopped by adding 2M sulphuric acid. Absorbance was measured using a 96well plate reader at 450nm.

2.8.3 Optimisation of Direct ELISA protocol

2.8.3.1 Adsorption of antibodies against E. coli

Antibodies were adsorbed against *E. coli* as per section 2.6.3 and used as per the protocol in 2.8.2.

2.8.3.2 Optimisation of blocking step

To determine the optimum concentration of milk powder within the washing buffer, the following concentrations of skimmed milk powder (Marvel, Sainsburys UK) within PBS containing 0.05% (v/v) Tween 20 were used; 0.1%, 1%, 5% (w/v).

2.8.3.3 Serial dilutions of bacteria to allow for testing of different ELISA conditions to optimise the immunoassay

To determine the detection level of the antibodies used within the ELISA, serial dilutions were undertaken. Cultures were prepared as in 2.8.1, however bacterial samples were prepared to 1 OD units at 600nm. Within a 96 well plate (Nuclon, flat), 50µl of carbonate bicarbonate buffer was added to all wells except those in column 1. Within column 1, 100µl of bacteria was added to the appropriate well and from column 1, a 1:2 dilution was completed to column 12 (Figure 2.1). The plate was incubated for 16-18hr at 37°C. Plates were then processed as described in section 2.8.2, unless otherwise stated.

The following controls were used;

- No bacteria control, where the bacterial sample was replaced with un-inoculated carbonate bicarbonate buffer
- a negative control, using *E. coli* as the bacterial sample

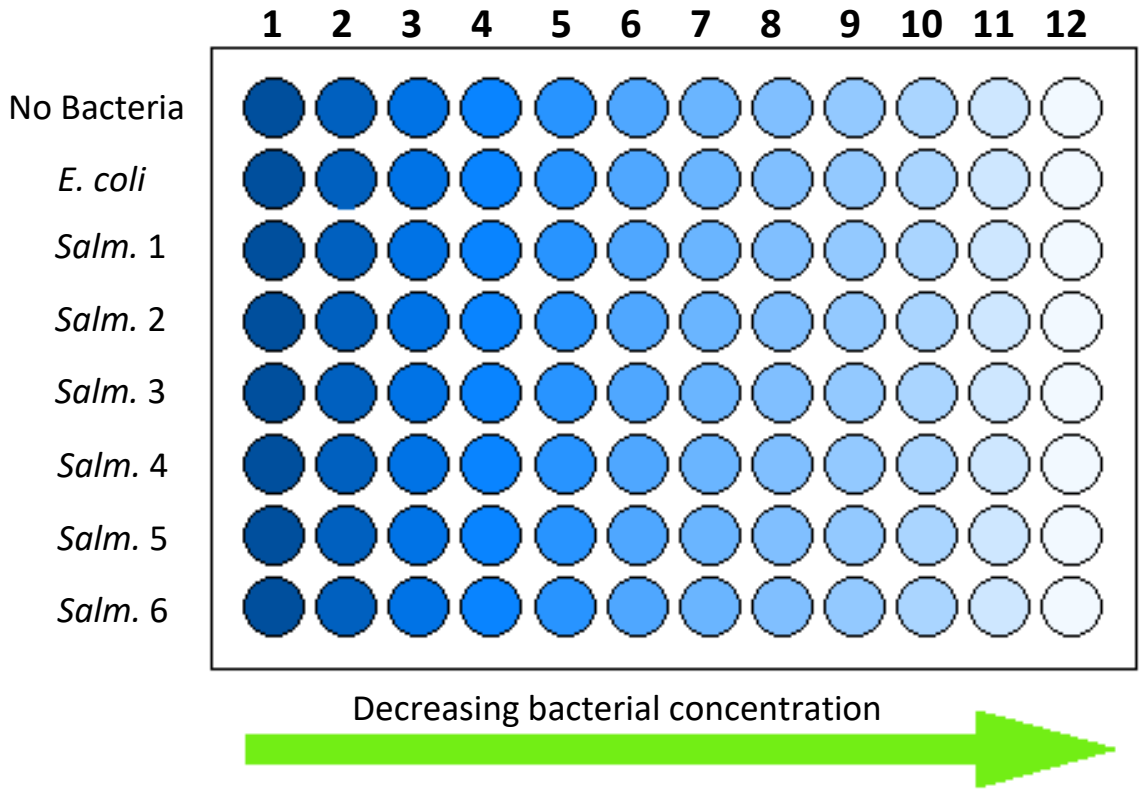

Figure 2.1: How a 96-well plate would be inoculated by serial dilution to allow for optimisation of multiple ELISA steps.

2.8.3.3.1 Optimisation of wash steps

To determine the effect of the wash step on the efficacy of Direct-ELISA, the protocol in section 2.2.8.2 was completed with the following changes to the wash steps. Firstly, washing was completed using a multichannel pipette, 200µl of PBS was added to each well, before plates were inverted and tapped dry on absorbent paper. Secondly, washing was completed using a plastic wash bottle containing PBS. PBS was squeezed over the plates, ensuring all wells were filled, before plates were inverted and tapped dry on absorbent paper

2.8.3.3.2 Optimisation of antigen incubation temperature

To determine the effect of incubation temperature of the antigen step the protocol in section 2.2.8.3 was completed with the following changes to antigen incubation temperature; incubation at 4°C and incubation at 37°C.

2.8.3.3.3 Optimisation of antibody concentration

To determine the effect of antibody concentration on the sensitivity of the ELISA, the protocol in section 2.2.8.3 was completed with the following changes to antigen concentration for the monoclonal antibody (A99H – Table 2.15); dilution at 1:100 and dilution at 1:500.

2.9. Potentiometric Vantix assays for the detection of *Salmonella* sp.

All immunoassays were completed in triplicate unless otherwise stated.

2.9.1 Preparation of antigen cultures for Vantix assays

Cultures were grown aerobically in 100ml nutrient broth within conical flasks on a shaking incubator at 37°C for 16-18 hours. Aliquots of 20ml of the culture was centrifuged at 5000rpm for 20mins. The resultant pellets, within falcon tubes, were then washed 3 times in 10ml PBS at 5000prm for 20mins. Pellets were then re-suspended in 10ml carbonate bicarbonate buffer and a stock solution of the required absorbance was prepared using a spectrophotometer. For this protocol the following controls were used;

- No bacteria control, where the bacterial sample was replaced with un-inoculated carbonate bicarbonate buffer
- a negative control, using *E. coli* as the bacterial sample

2.9.2 Assays using Vantix Diagnostic Research Tool 1 (VR1)

For initial assay development, the original Vantix Diagnostic Research Tool (VR1) was used (Figure 2.2).

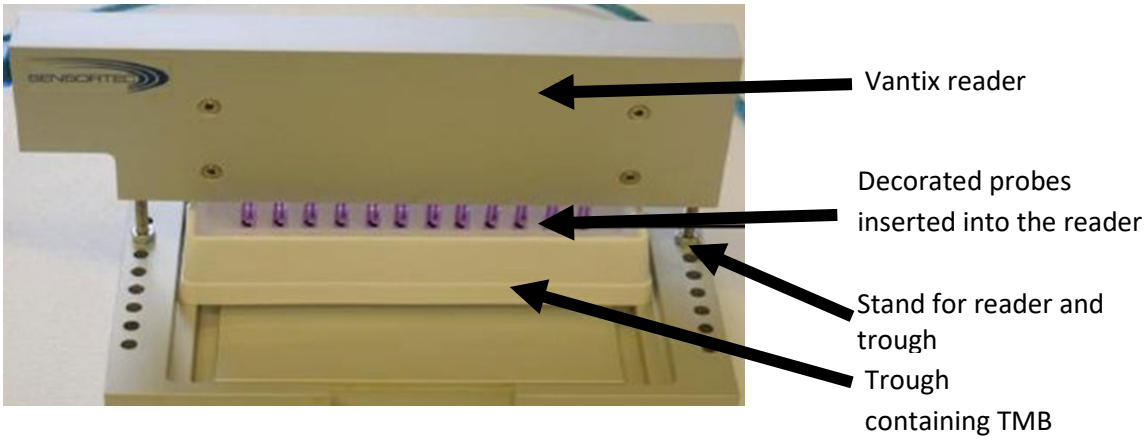

Figure 2.2: The Vantix Diagnostic Research Tool (VR1), which detects the potentiometric response of the assay on the decorated probes.

2.9.2.1 Probe preparation for assay using the VR1

Multiple probes are provided in long strips and thus were trimmed to allow for insertion into the head of the original Vantix Diagnostic Research Tool (VR1) (Figure 2.3). Silver reference electrodes were kept clear of all reagents throughout the experiment. When incubated all probes were kept within a moist environment to ensure that reagents did not dry.

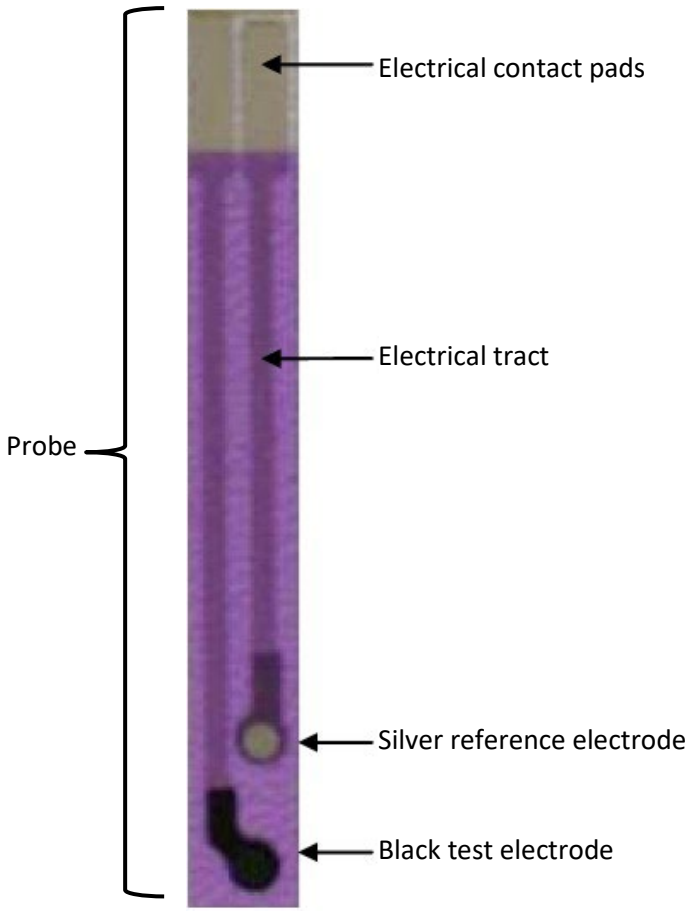

Figure 2.3: An example of a VR1 probe. The potentiometric signal generated by the assays is detected by the difference in voltage between the two electrodes (Image adapted from Cork *et al*., 2013).

2.9.2.2 Direct Vantix assay

Cultures were prepared as stated in section 2.9.1. On the black test electrodes of the sensors, Antigen/control aliquots of 5µl were added and probes were incubated at 37°C for 16-18hrs. Probes were washed by submerging and rinsing probes with PBS, then blotted dry. Black electrodes were blocked with 5µl of PBS containing 0.1% skimmed milk powder (w/v) for 1 hour at 37°C. Probes were washed and then aliquots of 5µl of conjugated antibody were added to the black electrodes. Probes were then incubated at 37°C for 2 hours. Probes were then washed three times and inserted into the reader of the VR1. Probes were then submerged into TMB substrate (section 2.7.3), enough to cover the silver reference electrode and read using the VR1.

Antibody incubation time was reduced, from 120 to 60 to 45 to 30 minutes to see if incubation time influenced antigen detection.

2.9.2.3 Sandwich Vantix assay

For the following assay polyclonal TRP was used as the capture antibody and monoclonal BMM was used as the detection antibody as detailed in table 2.15.

Cultures were prepared as stated in 2.9. On black test electrode, 3µl of unconjugated capture antigen was aliquoted and incubated for 1hr at 37°C. Probes were washed by submerging and rinsing in PBS, then blotted dry. On the black electrode of the probes, antigen/control aliquots of 3µl were added and probes were incubated at 37°C for 2hrs. Washing was repeated, and black electrodes were blocked with 3µl of PBS containing 0.1% skimmed milk powder (w/v) for 1 hour at 37°C. Probes were washed and then aliquots of 3µl of conjugated antibody were added to the black electrodes. Probes were then incubated at 37°C for 2 hours. Probes were then washed three times in PBS and inserted into the reader. Probes were then submerged into TMB substrate, enough to cover both electrodes and read using the VR1.

2.9.3. Assays using the Vantix Diagnostic Research Tool 2 (VR2)

For the following assays polyclonal TRP was used as the capture antibody and monoclonal A99H was used as the detection antibody, as detailed in table 2.15. The Vantix Diagnostic Research Tool (VR2) was used in the following assays (Figure 2.4).

Figure 2.4: The Vantix Diagnostic Research Tool 2 (VR2), an optimisation of the VR1, that detects the potentiometric response of immunoassays (Image adapted from https://www.egtechnology.co.uk/portfolio/vr2-assay-device).

2.9.3.1 Probe preparation for assay

Probes came in pre-designed combs to fit the VR2 (Figure 2.5). Silver reference electrodes were kept clear of all reagents throughout the experiment. When incubated all probes were kept within a moist environment to ensure that reagents did not dry.

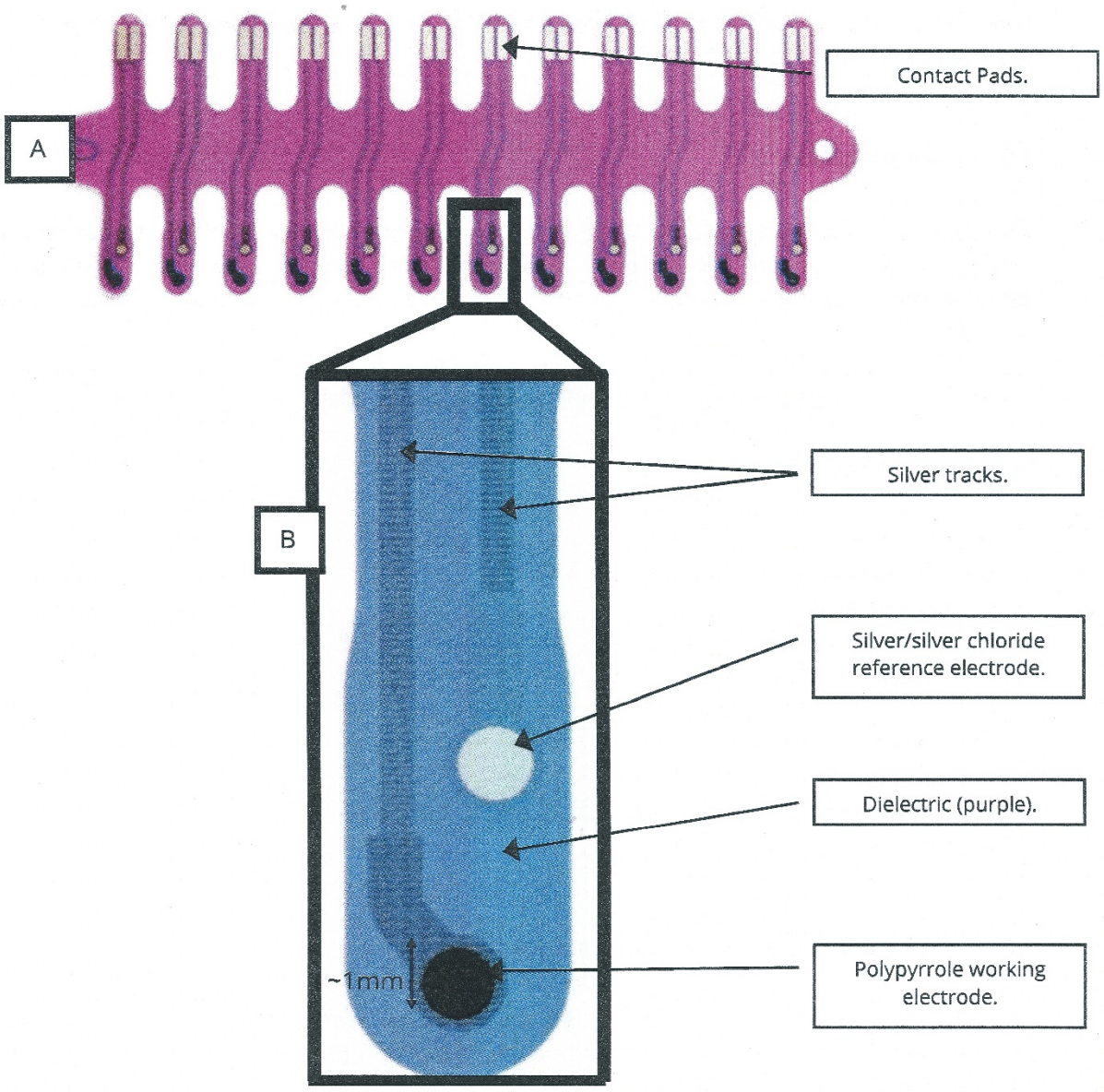

Figure 2.5: An example of a VR2 comb of probes. A = sensor comb consisting of 12 probes, B = An individual probe showing a close view of electrodes (Image taken from Vantix.com).

2.9.3.2 Sandwich Vantix assay

Cultures were prepared as stated in 2.9. On black test electrodes, 2µl of unconjugated polyclonal antibody (TRP, 1:500) was aliquoted then incubated at 37°C for 30 mins, Figure 2.6. The comb was then rinsed in PBS, avoiding wetting the silver reference electrodes, then blotted dry. On black test electrodes, 2µl of test sample/control was placed on the electrode and incubated for 60mins at 37°C.

Wash procedure was repeated, then black electrodes were blocked with 2µl 0.1% skimmed milk powder (w/v) and incubated at 37°C for 30mins. After washing, 2µl of conjugated monoclonal antibody (A99H, 1:500) was placed on to black test electrodes and combs were incubated at 37°C for 30mins. Washing procedure was then repeated in triplicate. The comb was then inserted into a clip (Figure 2.7) and the clip was inserted into the VR2 reader. Probes were submerged in TMB substrate to cover the silver electrode and read using the VR2.

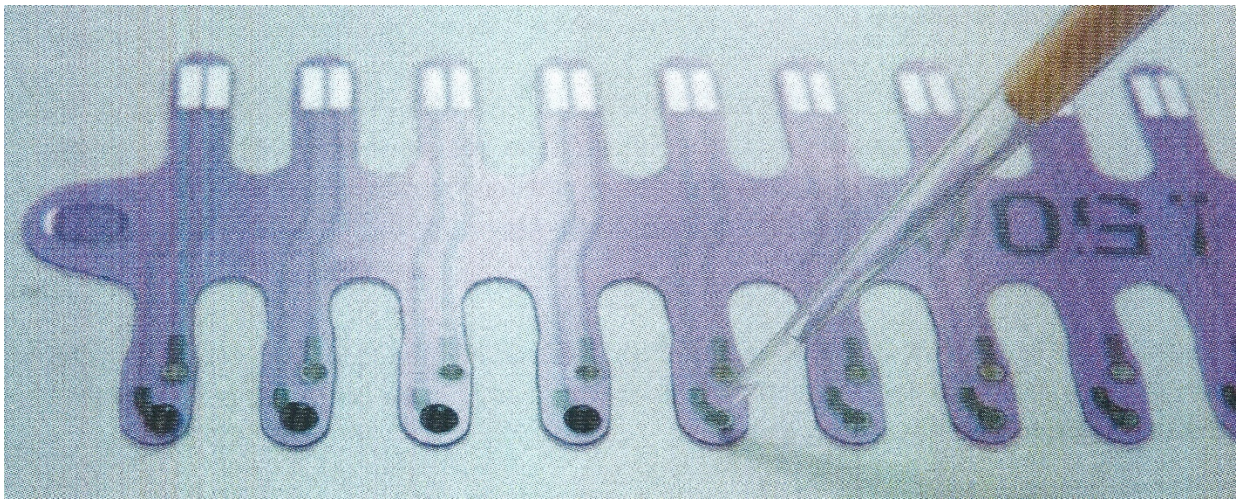

Figure 2.6: A comb of VR2 probes being decorated with an assay substrate (Image taken from Vantix.com).

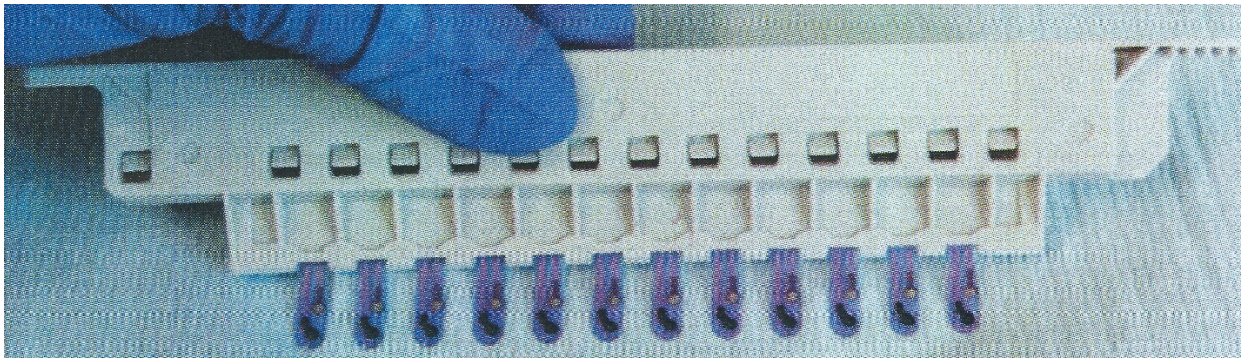

Figure 2.7: A clip containing decorated probes to be read within the VR2 reader (Image taken from Vantix.com).

2.9.3.2.1 Determination of detection antibody concentration

To determine if an increased concentration in monoclonal antibody (A99H) increased the sensitivity of the sandwich Vantix assay the protocol in 2.9.2.2 was used with the following variables: dilution of 1:100 for monoclonal antibody and dilution of 1:500 for monoclonal antibody

2.9.3.2.2 Reduction in step incubation time

Sandwich assays was completed as per section 2.9.2.2, however differing incubation times were tested as per Table 2.16 to determine if sandwich assay sensitivity could be maintained through shortened incubation periods

Table 2.16: Incubation times for optimising the VR2 Sandwich assay

Overall incubation time (hours)	Antibody and blocking incubation time (mins)	Antigen incubation time (mins)
2.5	30	60
2	30	30
1.5	15	60
1.25	15	30
1	15	15
0.66	10	10

2.9.3.3 Testing the specificity of the optimised sandwich assay

A panel of *Salmonella* sp. were tested alongside the controls. Cultures were prepared as stated in section 2.9.1. On black test electrodes, 2µl of unconjugated polyclonal antibody (TRP, 1:500) was aliquoted then incubated at 37°C for 15mins. The comb was then washed in PBS by dragging it back and forth, avoiding wetting the silver reference electrodes, then blotted dry. On black test electrodes, 2µl of test sample/control was aliquoted and incubated for 30mins at 37°C. Wash procedure was repeated, then test electrodes were blocked with 2µl 0.1% skimmed milk powder (w/v) and incubated at 37°C for 15mins. After washing, 2µl of conjugated monoclonal antibody (A99H, 1:100) was aliquoted on to black test electrodes and combs were incubated at 37°C for 15mins. Washing procedure was

then repeated in triplicate. The comb was then inserted into a clip and the clip was inserted into the VR2 reader. Probes were submerged in TMB substrate to cover the silver electrode and read using the VR2.

2.9.3.4 Vantix sandwich assay through calf scour

Sandwich assays were completed as described in section 2.9.2.3. Additionally, sandwich assays were completed with faecal samples, containing known bacterial numbers, used in place of antigen.

Faecal samples were treated as per section 2.1.2 before use in the Vantix sandwich assay. Faecal samples were vortexed to ensure a uniform suspension of matter. Bacteria were prepared to the appropriate absorbance as per the method stated in 2.8.1. Using a sterile microcentrifuge tube, aliquots of 90µl of scour along with 10µl of bacterial suspension were mixed to create a spiked positive sample.

For a 1:2 dilution of faecal matter, 50µl of faecal matter was added to 40µl of PBS and 10µl of bacterial suspension.

3. Bioinformatics

3.1 Bioinformatics to identify and target pan-*Salmonella* genes

Cheung and Kam (2012) note that conventional culture methods require a long turnaround time and, if most samples are negative, time, labour, and resources are wasted (Falkenhorst *et al.*, 2013). To significantly reduce required resources and enhance efficiency, rapid methods for *Salmonella* detection are needed to improve laboratory service and epidemiological studies, as well as the diagnosis and prognosis of cattle suffering from scour (Cheung and Kam, 2012; Kingsley *et al*., 2009). Molecular diagnostics are routine within laboratories due to high sensitivity and specificity, as well as being relatively quicker than culture, however, to ensure that the efficacy, sensitivity, and specificity of molecular techniques are maintained, the DNA sequences targeted need to be specific to the only target pathogen (Cheung and Kam, 2012; Kingsley *et al*., 2009). To do this bioinformatics is employed to determine conserved sequences.

Bioinformatics applies computer science, applied mathematics and statistics to the study of biology and has become widespread within microbiology due to the use of whole genome sequencing, (Carrico *et al*., 2018). Bioinformatic tools have been instrumental in sequence processing and gene discovery with software available to create phylogenetic trees, generate primers for microbial detection, and align sequences to detect homology amongst genes/genomes (Carrico *et al*., 2018).

When aligning genomes, homologous nucleotides across two or more genomes are sought (Darling *et al*., 2010). Within this study the genome alignment tool ProgressiveMauve was utilised. Mauve uses comparative genomics, accepting each genome as a mosaic of unique lineage; specific segments, regions shared with a subset of other genomes, and segments conserved among all the genomes under consideration (Darling *et al*., 2010). The program relaxes' the assumption that genomes are all collinear in favour of local collinearity. Local collinearity is represented by LCBs (Local Collinear Blocks), which are homologous regions shared by two or more genomes within the alignment (Darling *et al*., 2010).

Darling *et al.* (2004) state that Mauve integrates the analysis of large-scale evolutionary events with traditional multiple sequence alignment, making it highly sensitive, as well as accurate and easy to use. The original Mauve model does not account for gene duplication and loss that can frequently occur in enterobacteria: factors that may alter patterns of genome evolution are not modelled for (Darling *et al*., 2010). Darling *et al*. (2010) developed ProgressiveMauve to account for this.

With ProgressiveMauve, Darling *et al*. (2010) build upon the features of the original Mauve model, aligning conserved regulatory regions and hypervariable intergenic regions, demonstrating a substantial increase in accuracy. Genomes that have undergone mutations resulting in the loss, rearrangement or gain of genes are now addressed, increasing the robustness of the tool (Darling *et al*., 2010). Additional work has gone into enabling clear visualisation of alignments, with easy visualisation of genome rearrangement, patterns of segmental gain/loss, and local conservation of nucleotide sequences (Darling *et al*., 2010).

Polymerase Chain Reaction (PCR) and Loop-mediated isothermal AMPlification (LAMP) are nucleic acid amplification techniques. PCR utilises a set of two primers, one forward, one reverse, that target the desired DNA sequence and through thermal cycling and thermostable *Taq* polymerase, make multiple copies of the target DNA to allow for detection. LAMP utilises a set of six primers, three forward primers, three backward primers, that target the desired sequence and through a polymerase with strand displacement activity, typically *Bst* polymerase, facilitates cyclic amplification that can occur at a single temperature.

To ensure understanding within the context of the current study, several terms have been defined to ensure clarity of terminology;

- Homology amongst sequences is defined as areas of nucleotide similarity greater than can be explained by pure chance.
- Conserved sequences are areas of homology that have been found across multiple *Salmonella* sp. genomes, either within this study or within relevant literature.

- Collinear is defined as points lying in the same straight line
- Specificity of conserved areas of genome/sequence is nucleotide similarity of the target area to other *Salmonella* sp.
- Specificity of primer sets is defined as the nucleotide similarity of a primer set to a genome, whether it is the target organism or not.
- Highly specific sequences/primer sets are those with nucleotide similarity to *Salmonella* sp. only
- A match within a primer set was considered as a match to a genome.
- An unintentional match was considered as a match within the *Salmonella* genome but outside of the targeted sequence.
- A non-specific match was considered as a match not within the *Salmonella* genome.

The aim of the bioinformatics used within this study is to generate primers for PCR and LAMP assays. To achieve this the following objectives were set;

- Identify previously published and sequenced genomes of *Salmonella enterica* serovars and align them utilising MAUVE.
- Using the MAUVE genome alignment, and relevant literature, identify genes that are highly conserved across multiple genomes.
- To ensure specificity to multiple *Salmonella enterica* serovars only, use BLASTn to align and verify specificity of the highly conserved genes found.
- Use specific and highly conserved genes to develop PCR primers utilising Primer BLAST.
- Use gene areas targeted by the developed PCR primers to develop LAMP primers utilising Primer explorer V.4.

3.2 Expanded methods for bioinformatics techniques

The general methodology of the bioinformatic tools used within this chapter are detailed within section 2.2

3.2.1 Mauve: multiple alignment software

Genomes were aligned using Mauve, (Darling *et al.*, 2010; Darling *et al.*, 2004). A full alignment within ProgressiveMauve was used with default parameters and sequences were entered in a Genbank format. Once aligned, the sequences were screened manually for highly conserved areas of sequence.

Mauve uses the first genome entered as the reference genome by default, however by selecting the R button alongside each genome this can be set by the user. Whilst aligning the genomes Mauve identifies conserved segments that appear to be internally free from genome rearrangements, which are referred to as Locally Collinear Blocks (LCBs) and can be seen in Figure 3.1. as the coloured blocks sectioned along the genome.

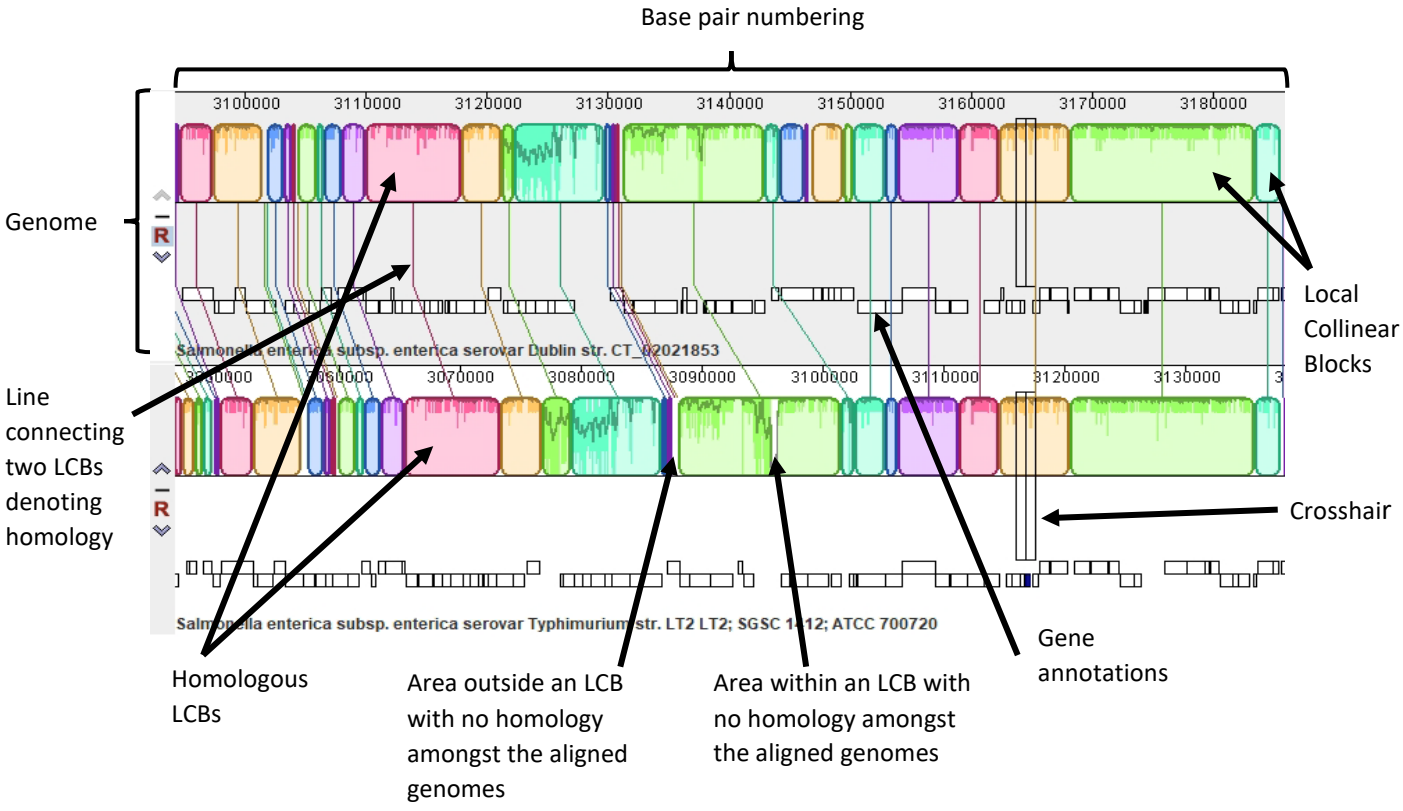

Figure 3.1: A section of a Mauve alignment, showing 2 genomes, *S.* Dublin and *S.* Typhimurium LT2 with annotations of features within a Mauve alignment

Each genome is displayed horizontally along a central line, LCBs above the line run in the same orientation as the reference genome, whilst LCBs below the line run anti-sense to the reference genome, these represent inversions of the genome segment (LCB) relative to the reference genome. Gaps outside of LCBs are areas of genome specific sequence that are not homologous with the aligned genomes. Within the LCBs are similarity profiles, seen in Figure 3.1 as the coloured line within the blocks. The similarity profile shows the average levels of conservation in that region of the profile. Any white areas within LCBs are specific to that genome only, within the alignment. Between the genomes, connecting LCBs, are lines, which indicate regions in which the genomes are homologous to each other.

Beneath the LCB alignment is a row that displays coding sequences (CDS), defined within the Genbank file, shown as white boxes. In parts of the display these boxes are red for rRNA genes or green for tRNA genes. CDS features indicate the range of a coding sequence for a protein, normally from an initiation codon to a termination codon. By hovering over these within the alignment output, gene names and annotation can be seen, as well as links to NCBI Entrez protein entries. By using the cross hair particular LCBs/genes can be aligned across the genomes to allow for observation of homology among multiple genomes (http://darlinglab.org/mauve/mauve.html).

Manual screening of the Mauve alignment was achieved by enhancing the alignment and identifying LCBs with a high degree of similarity (i.e. high colour intensity and little or no white areas) present across all 12 aligned genomes. From this, the CDS features associated with the locus of the LCB were checked for information of gene sequence within the NCBI database. Available gene sequences were collected from the NCBI database in FASTA format.

3.2.3 PrimerExplorer V.4: LAMP primer design software

For LAMP Primer specifications and base methodology, see section 2.4.4.2

To generate LAMP primers within PrimerExplorer V4. sequences were entered in text file format at 2,000bp maximum. Once entered, if the sequence is accepted for primer development a '*' will be displayed. Once generated, a large graphic shows the potential sets available.

3.2.3.1 Designing base LAMP primers

For the purpose of this study, base LAMP primers were defined as the FIP/BIP and F3/B3 primers, 4 primers in total, as seen in Figure 3.2.

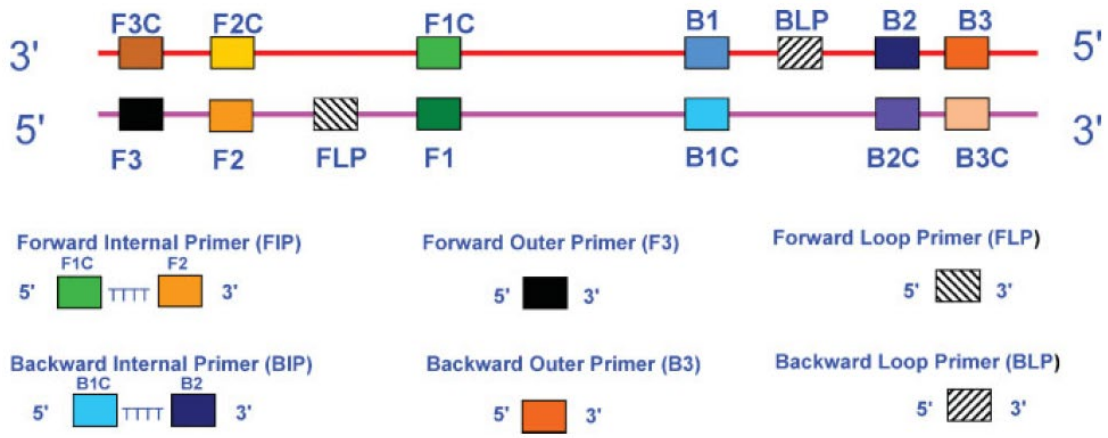

Figure 3.2: Schematic representation of a complete LAMP primer set, including loop primers and their positioning on that target DNA (Taken from Parida *et al*., 2008).

To generate base LAMP primers, PrimerExplorer V4 was used with the default design option selected, allowing the design of regular primers. Other options are available to target specific mutations if creating primers targeting wildtype/mutant DNA. Automatic judgment of the sequences was used, determining sequence GC content, and adjusting temperature, primer set length and GC content parameters for the user. Automatic judgement determines GC content; AT rich if GC content ≤45%, GC rich if GC content was ≥60%, standard parameters if GC content was between 45% - 60%, unless otherwise stated. PrimerExplorer V4 aims to generate primers with a GC content between 40-65%, as this has been determined to give good LAMP primers (Notomi *et al*., 2000).

Before developing LAMP primers, PCR primers were developed and tested to show target genes identified as pan-*Salmonella* by bioinformatics could be determined experimentally. The sequence

targeted by the PCR primers (see table 2.2 for nucleotide positioning within the genes) were selected, using the 'select range' feature placing F2-B2 within the PCR target sequence range. In cases where this provided unsuitable primer sets, due to incomplete/unstable primer sets, 'ignore range' was chosen, allowing for LAMP primer sets to be generated along the entire gene sequence. Within 'mutation/consensus' settings, 'no mutations' were selected, as this work is targeting conserved areas of the *Salmonella* sp. genome.

PrimerExplorer V4. generates LAMP primers to allow for the most specific, stable and sensitive primer set, the following are adjusted to allow for this;

- Melting temperature (Tm) – designed to be 65°C (±1C) for F1c/B1c and loop primers, about 60°C (±1C) for F2/B2 and F3/B3
- Secondary structures (primer dimers) – the program automatically detects primers and primer sets possessing complementary sequences and eliminates them, otherwise if the primers interact within a set, the LAMP reaction can be slowed or halted. Extra attention is paid to ensure 3' ends are not complementary.
- Distance between primers – Primers are spread along the sequence to enable correct recognition, reading, and replication of the target genome. Between the 5' end of F2 and B2, 120-180bp. Between F2 and F3/ B2 and B3, 0-20bp. Between the loop forming regions, 5' end of F2 to 3' end of F1/ 5'end of B2 to 3' end of B1, 40-60bp.
- Primer end stability – The 6bp at each terminal of a primer were considered primer ends, which serve at starting points of DNA synthesis and are required to be stable. This is measured in ΔG, the change in free energy. The change in free energy is the energy of a system that occurs during a reaction, which is calculated by the difference between product free energy and reactant free energy. Generally, a reaction will proceed in the direction that causes ΔG to be zero. The more negative primer ΔG is, the more often the primer will anneal

to the template DNA. Thus 3' ends of F2/B2, F3/B3, and LF/LB and the 5' end of F1c/B1c are designed so that ΔG ≤ -4kcal/mol.

Candidate primers were generated, then by selecting 'Display' the first 100 primer sets generated and their position on the target sequence will be shown. Sets can be sorted by;

- **Dimer** – ascending formation of secondary structures
- **F2 5' position** – distance between 5'end of target sequence and F2 5' end
- **F2-B2 range** – length between F2 and B2 from short to long
- **5'/3' edge stability** – ascending end stability
- **Random** – sets are displayed randomly
- **None** – order of generation

A maximum of 1000 primers can be generated and lists the top 100 based on requested sort order. The full dataset for selected primers was collected, then used to design loop primers. When selecting primer sets, the ΔG of the primer set was required to be ≥ -9.0kcal/mol, ensuring the primer set was stable, with a reduced chance of primer dimers (Parida *et al.,* 2008). Additionally, primer sets with 3' ends that were GC rich were preferentially chosen, to ensure primer stability.

Primer sets were entered into BLASTn was used to determine specificity, by searching against both *Salmonella* and non-*Salmonella* genomes two primer sets, that best fit the specifications set in section 2.4.4.2, were chosen per PCR target sequence.

3.2.3.2 Designing loop LAMP primers

From the selected LAMP primer set, the save file is opened within Primer design V4. Design options and GC content could not be changed, as they were set by the program to match the regular primer set, however all other parameters could be adjusted, including the length of FLoop/BLoop primers (Figure 3.2), and were treated as in section 3.2.3.1 Loop primer information was saved. Where loop

primers were not generated, parameters were adjusted and if no results were yielded, a different regular primer set was chosen and used instead.

Once complete LAMP primer sets were generated (base and loop primers), sets were screened for specificity and unwanted matches (see section 3.2.2) and sent for synthesis (see section 2.3.3).

3.3 Bioinformatic results

3.3.1 Genome selection for Mauve alignment

A total of 12 *Salmonella enterica* genomes were acquired from National Centre for Biotechnology Information (NCBI) microbial genome resources as seen in Table 3.1, and analysed with methodology detailed in section 2.2.1

Table 3.1: *Salmonella enterica* genomes collected to align in Mauve

*host adapted to this organism, can still infect other organisms

***Salmonella* serovar**	**Strain code**	**Strain Reference**	**Host organism***	**GenBank accession no.**
S. Choleraesuis	SC-B67	*S.* Choleraesuis	Pig	NC_006905
S. Dublin	CT 02021853	*S.* Dublin	Cow	NC_011205
S. Enteritidis	EC20121176	*S.* Enteritidis (2012)	Non-specific	CP007270
S. Enteritidis	P125109	*S.* Enteritidis (P1)		NC_011294
S. Gallinarum	287/91	*S.* Gallinarum (287)	Chicken	Nc_011274
S. Gallinarum	RKS5078	*S.* Gallinarum (RK)		NC_016831
S. Newport	SL254	*S.* Newport (SL)	Cow	NC_011080
S. Newport	USMARC-S3124.1	*S.* Newport (US)		NC_021902
S. Typhi	CT18	*S.* Typhi (CT)	Human	NC_003198
S. Typhi	Ty2	*S.* Typhi (Ty2)		NC_004631
S. Typhimurium	LT2 (ATCC 700720)	*S.* Typhimurium (LT2)	Non-specific	NC_003197
S. Typhimurium	14028S	*S.* Typhimurium (14)		NC_016856

3.3.2 Genomic alignment via Mauve to detect highly conserved genes

To identify conserved gene targets for the detection of pan-*Salmonella* sp., multiple *Salmonella* sp. needed to be analysed. To identify segments of sequence that were conserved across multiple *Salmonella* genomes, genome alignment was completed using Mauve (section 2.2.2). For figures 3.3, 3.4, 3.6 – 3.8, the genomes are ordered as such; *S.* Dublin, *S.* Newport (SL), *S.* Typhimurium (14), *S.* Typhimurium (LT2), *S.* Choleraesuis, *S.* Gallinarum (287), *S.* Typhi (CT), *S.* Typhi (Ty2), *S.* Gallinarum (RK), *S.* Enteritidis (P1), *S.* Newport (US), *S.* Enteritidis (2012). *S.* Dublin was used as the reference genome for the alignment, the remaining genomes were arranged so that the lines connecting LCBs lay in as linear a pattern as possible. For strain information see Table 3.3. For all Mauve alignments, *S.* Dublin was used as the reference strain. For information on how to interpret a Mauve alignment, see section 3.2.1

It can be seen in Figure 3.3 that *Salmonella* sp. have homologous sequences amongst different serovars, noted by vertical lines linking the aligned genomes. The 'noise' of the LCB lines within the alignment in Figure 3.3 could be due to seed weight. Mauve determines seed weight based on genome size, increasing seed weight can reduce this noise, however it may reduce the sensitivity of local collinearity, therefore seed weight was not adjusted.

A partial screening of the genomic alignment was completed. In total 32 conserved genes were identified, 11 of which were considered highly specific to multiple *Salmonella* strains and therefore potential primer targets (Table 3.2). From these, three were picked for primer development; *hilA, orgA,* and *bapA. hilA* and *orgA* are associated with SPI 1, a highly conserved segment across multiple *Salmonella* sp. and *bapA* is associated with biofilm formation.

In figure 3.5, *hilA* is shown as the white rectangle spanning from the orange LCB to partway through the pink LCB on 11 *Salmonella* serovars. Within the LCB's themselves, little variation between the serovars can be seen, noted by the solid colouring and regular plateau at the top of the blocks, indicating that *hilA* is highly conserved. Most genome annotations note that *hilA* codes for an invasion

protein regulator, with *S.* Newport (US) and Typhimurium (LT2) describing as a transcription regulator (Table 3.3). Where annotated, the LCB's within *S.* Enteritidis (2012) that align with the locus for *hilA* within the other genomes are described mostly as a hypothetical protein, likely due to the fragmented sequencing of the genome (Figure 3.3a).

In total, 32 conserved genes were identified, 11 of which were considered highly specific to multiple *Salmonella* strains and therefore potential primer targets (Figure 3.6). A partial screening of the Mauve genome alignment was completed. The genes *hilA*, *orgA,* and *bapA* were chosen as primer targets as they are highly conserved across multiple *Salmonella* species with sparse differences in sequence and *Salmonella* specific when analysed using BLAST.

Certain genes were removed from the pool of potential targets due to literature research, for example, Liu *et al.* (2010) note that *rfb* is not present in *S.* Choleraesuis, which is host adapted to pigs. Whilst not of primary interest to this study, a good primer target is as inclusive across species as possible whilst maintaining specificity. As other highly specific gene-targets were found that included *S*. Choleraesuis, *rfb* was screened out. Additionally, when the CDS data was utilised to discover a sequence within the NCBI database, *rfb* CDS data was only available for *Streptomyces coelioolers*, suggesting an immediate match outside *Salmonella* sp. In genes where CDS was not available in *Salmonella* sp., these genes were screened out. When originally checked, Jan 2015, *leuO* sequence was not available via the NCBI database, however a recent check carried out in September 2019, revealed that this sequence was now available within *S*. Dublin genome.

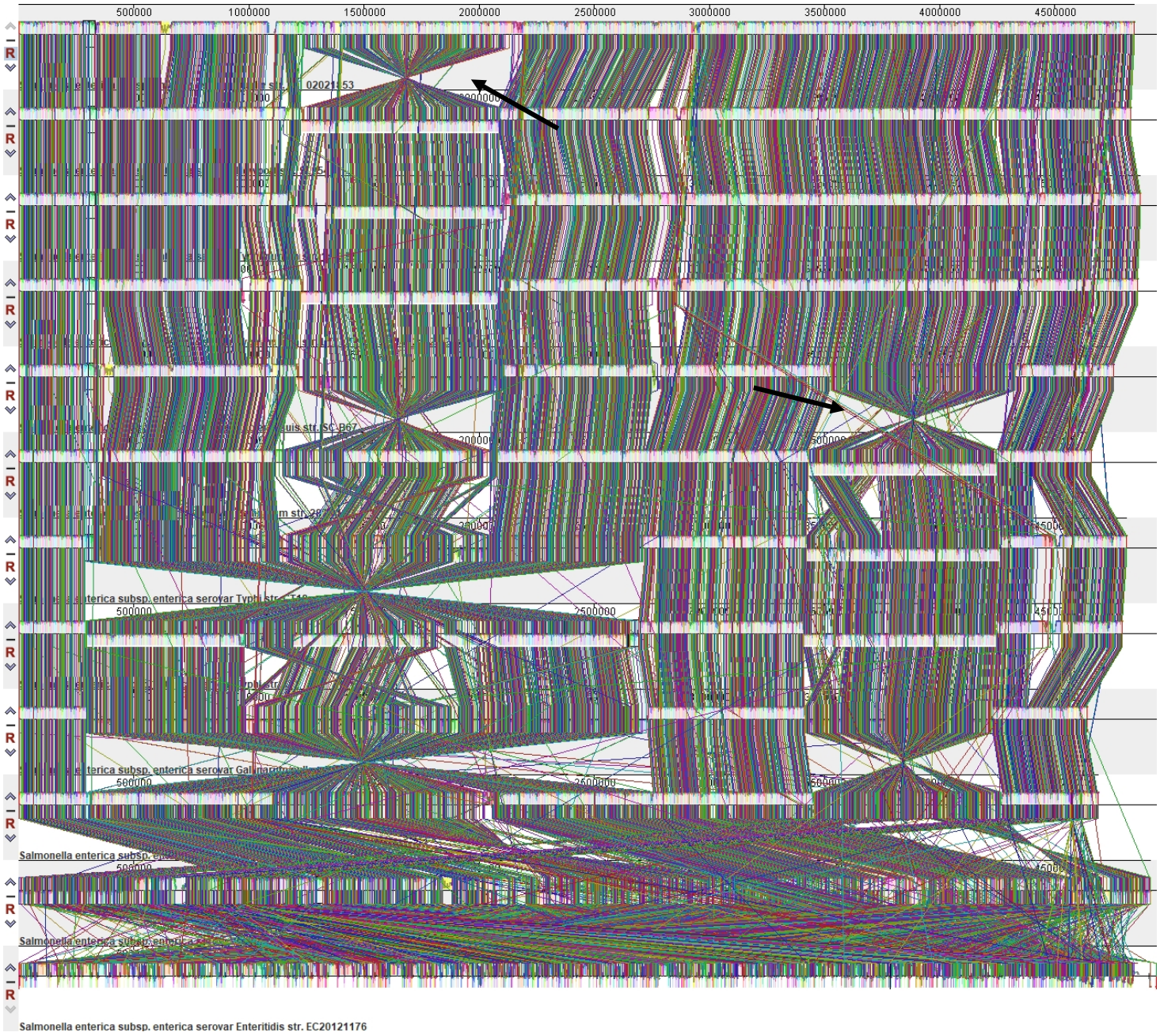

Figure 3.3: Alignment of 12 complete *Salmonella* genomes in Mauve. The vertical lines connecting LCBS denote sequence homology across multiple genomes within the alignment. Several potential 'inversions' in sequence structure can be seen, black arrows show two examples.

Figure 3.4: Alignment of 12 complete *Salmonella* genomes in Mauve, as seen in Figure 3.2, without the lines connecting the LCB's.

Table 3.2: Genes selected via Mauve genome alignment and screened via BLASTn for potential matches to organisms other than *Salmonella* sp.

Key: Y = yes, X = no

Gene	Gene sequence collected from NCBI	BLASTn sequence match (base pair number)	Unwanted matches	Potential target gene
bapA (stm2689)	Y	None		Y
citg	Y	>=200		X
clpX	Y	>=200		X
cyoD	Y	>=200	*E. coli* *Shigella* sp.	X
frr	Y	>=200		X
gpt	Y	>=200		X
hilA	Y	None		Y
hilC	Y	None		Y
hupB	Y	>=200	*Citrobacter* sp. *Escherichia* sp.	X
invA	Y	None		Y
invH	Y	None		Y
IspA	Y	>=200		X
leuO	not available via NCBI database	-	-	X
lon	Y	>=200	*Citrobacter* sp. *E. coli*	X
oafA	Y	>=200	*Enterobacter cloacae*	X
orgA	Y	None	None	Y
orgB	Y	None	None	Y
pdhR	Y	>=200	*Citrobacter* sp. *E. coli* *Enterobacter* sp.	X
pdxA	Y	>=200	*E. coli* *Shigella* sp.	X
ppD	Y	>=200	*E. coli*	X
prgK	Y	None	None	Y
rfb	CDS comes up as *Streptomyces coelioolers*	-	-	X
rrs	CDS only available in *Chloroflexus aurantiacus*	-	-	X
smPB	Y	>=200	*Citrobacter* sp. *E. coli* *Enterobacter* sp. *Shigella* sp. *Serratia* sp.	X
stn	Y	None		Y
ssaK	Y	None		Y
xseB	Y	>=200	*Citrobacter* sp. *E. coli* *Shigella* sp. *Serratia* sp.	X
yabB	Y	>=200	*Citrobacter* sp. *E. coli*	X
yacG	Y	None	None	Y
yadF	Y	>=200	*Citrobacter* sp. *E. coli*	X
yaiY	Y	80-200		X
yejM	Y	>=200	*Citrobacter* sp. *E. coli* *Shigella* sp.	X

Figure 3.5: Magnified section of the alignment in Fig 3.3, showing the locus of homologous sequence associated with *hilA* which is denoted by the gene annotations within the white box underneath the sequences of the aligned genomes. The vertical lines connecting LCBs of matching colours show sequence homology across the genomes, this figure shows the alignment focused upon the turquoise LCB of the *hilA* gene.

Figure 3.6: Magnified section of the alignment in Fig 3.3, showing the locus of *orgA* on the 12 *Salmonella* genomes. The black arrows show the coding sequence annotations, within the white box for the locus of *orgA*, which is contained within an orange LCB of homology.

Figure 3.7: Magnified section of the alignment in Fig 3.3, showing the locus of *bapA* denoted as the gene annotation within the white box under the sequences beneath the different coloured LCBs, within the 12 genome alignment.

In figure 3.6, *orgA* is shown as the white rectangle within a large orange LCB. As with *hilA* little variation between serovars can be seen, suggesting *orgA* is highly conserved. The genome annotations refer to *orgA* as both a cell invasion protein and an oxygen-regulated invasion protein. *S*. Typhimurium (14) refers to *orgA* as a needle complex assembly protein, whilst *S.* Dublin and *S.* Newport (SL) refer to *orgA* as a type III secretion apparatus protein (Table 3.4).

In figure 3.7, *bapA* is shown, where annotated, as the white rectangle spanning from the end of the orange LCB to partway through the pink LCB on 11 *Salmonella* genomes. Within the LCBs there is slightly more variation that seen in *hilA* or *orgA*, however there is mostly solid colouring and a plateau at the top of the blocks. At the locus of *bapA,* 5/11 genomes do not note a gene, represented by the white rectangles under the genome, and where a gene is proposed, it is often considered a pseudo gene or a large repetitive protein (Table 3.5). As with *hilA*, *S.* Enteritidis (2012) has LCBs that will align with sequences within the genomes, however at differing loci, and were annotated refer to these fragments as a hypothetical protein (Table 3.5a).

Table 3.3: The coding sequence information gathered for the gene/locus code associated with *hilA,* including the gene annotation and potential protein purpose, for the aligned genomes.

***Salmonella* serovar**	**Gene/locus code**	**Proposed purpose**
Dublin	seD_A3186	Invasion protein regulator
Choleraesuis	*hilA*	Invasion protein regulator
Enteritidis (2012)	See Table 3.4a	See Table 3.4a
Enteritidis (P1)	*hilA*	Invasion protein regulator
Gallinarum (287)	*hilA*	Invasion protein regulator
Gallinarum (RK)	*hilA*	Invasion protein regulator
Newport (SL)	SNSL254_A3081	Invasion protein regulator
Newport (US)	SN31241_39680	Transcription regulator *hilA*
Typhi (CT)	*iagA*	Invasion protein regulator
Typhi (Ty2)	*iagA*	Invasion protein regulator
Typhimurium (14)	*hilA*	Invasion protein regulator
Typhimurium (LT2)	*hilA*	Transcription regulator *hilA*

Table 3.3A: The positioning of the conserved sequence segments within *S.* Enteritidis (2012) that are linked to parts of *hilA* sequences via LCB lines within the Mauve genome alignment, with gene annotation, where available.

Colour of LCB *	Base pair positioning	Locus tag	Propose purpose
Orange	4566899 – 4567210	AV71_29025	Hypothetical protein
Green	4651629 – 4651800	AV71_29900	Hypothetical protein
Turquoise	4748281 – 4748730	AV71_30640	Hypothetical protein
Blue	4543765 – 4544116	AV71_28845/ AV71_28845	Virulence protein/hypothetical protein
Purple	4813612 – 4813815	AV71_3105	Hypothetical protein
Pink	3065915 – 3065660	-	-

* as seen in figure 3.5

Table 3.4: The coding sequence information gathered for the gene/locus code associated with *orgA,* including the gene annotation and potential protein purpose, for the aligned genomes.

***Salmonella* serovar**	**Gene/locus code**	**Proposed purpose**
Dublin	SeD_A3180	Type III secretion apparatus protein OrgA/MxiK
Choleraesuis	*orgA*	Inner membrane protein
Enteritidis (2012)	AV71_14980	Oxygen-regulated invasion protein OrgA
Enteritidis (P1)	SEN2712	Cell invasion protein
Gallinarum (287)	SG2773	Cell invasion protein
Gallinarum (RK)	SPUL_2867	Cell invasion protein
Newport (SL)	SNSL254_A3075	Type III secretion apparatus protein OrgA/MxiK
Newport (US)	SN31241_39620	Oxygen-regulated invasion protein OrgA
Typhi (CT)	*orgAa*	Cell invasion protein
Typhi (Ty2)	*orgA*	Oxygen-regulated invasion protein
Typhimurium (14)	*orgA*	Needle complex assembly protein
Typhimurium (LT2)	*orgA*	Oxygen-regulated invasion protein OrgA

Table 3.5: The coding sequence information gathered for the gene/locus code associated with *bapA,* including the gene annotation and potential protein purpose, for the aligned genomes.

***Salmonella* serovar**	**Gene/locus code**	**Proposed purpose**
Dublin	SeD_A3017	VCBS repeat containing protein
Choleraesuis	SC2689	Pseudogene = unknown
Enteritidis (2012)	See Table 3.6a	See Table 3.6a
Enteritidis (P1)	SEN2609	Hypothetical protein
Gallinarum (287)	SG2666	Pseudogene = true
Gallinarum (RK)	SPUL_2576	Pseudogene = true
Newport (SL)	SNSL254_A20903	Hypothetical protein
Newport (US)	SN31241_38010	Large repetitive protein
Typhi (CT)	STY2875	Large repetitive protein
Typhi (Ty2)	T2643	Large repetitive protein
Typhimurium (14)	STM14_3297	Pseudogene = true
Typhimurium (LT2)	STM2689 (Gene ID: 1254212)	Pseudogene; frameshift, proline/threonine rich protein

Table 3.5A: The positioning of the conserved sequence segments within *S.* Enteritidis (2012) that are associated via Mauve genome alignment with *bapA,* as well as gene annotation, where available.

Colour of LCB *	**Base pair positioning**	**Locus tag**	**Propose purpose**
Green	3081652 – 3087482	-	-
Turquoise	4291305 – 4293582	AV71_26675	Hypothetical protein
Blue	2211642 – 2213508	-	-
Purple	4440528 – 4440878	AV71_27945	Hypothetical protein
Pink	22747793 – 2748454	AV71_16365	Hypothetical protein

* as seen in figure 3.7

3.3.3 Generation of PCR Primers for detecting *Salmonella* sp.

From *hilA, orgA,* and *bapA*, 5 PCR primer sets per gene were generated using Primer BLAST (NCBI, methods section 2.4.4), from those 5 primer sets, 2 per gene were selected for experimental use (Table 3.6). The primer sets were screened through BLASTn to determine non-specific matches for organisms other than *Salmonella* sp. (Table 3.7). Whilst matches that would be relevant to the cattle industry were found for *hilA* and *orgA*, the primer sets were aligned against the genomes of the matches and screened out, either due to the positioning of the primers being divergent to each other or positioned too far apart to generate a product under the cycling conditions used.

Table 3.6: PCR Primer sets produced for the detection of *Salmonella* sp.

Key: c = complementarity, GC% = guanine/cytosine content of sequence

Lab I.D	Product Length (bp)	Primer type	Sequence (5'-3')	Tm (°C)	GC%	Self 5' c	Self 3' c
HilA_1FP	660	Forward	CGACAGAGCTGGACCACAAT	60.04	55	5	2
HilA_1BP		Backward	TCAAGCGGGGATCCTGTTTC	60.04	55	6	2
HilA_2FP	344	Forward	ACCAACCCGCTTCTCTCTTG	59.96	55	3	0
HilA_2BP		Backward	ATTGTGGTCCAGCTCTGTCG	60.04	55	5	2
OrgA_1FP	384	Forward	GCGGCGGCAAATGAGTTAAT	59.90	50	4	2
OrgA_1BP		Backward	AGCATCCTGCTTCAATGCCT	60.03	50	5	2
OrgA_2FP	437	Forward	TATCCATCCTCAGCGGTTGC	59.89	55	5	3
OrgA_2BP		Backward	CCTGCTTCAATGCCTCCTCA	60.03	55	3	1
BapA_1FP	425	Forward	CGGTGAATTCGTCGTTACGC	59.98	55	6	3
BapA_1BP		Backward	GATCGACAGTGATCCCGACC	59.97	60	7	2
BapA_2FP	591	Forward	ATCGGCAATAATGGCGCAAC	59.97	50	6	2
BapA_2BP		Backward	GATTTCATTGACGACGGGCG	59.97	55	5	3

Table 3.7: The matches generated to organisms other than *Salmonella* sp. by both the forward and reverse primers within a PCR set, when run through BLASTN.

Primer set	Latin name	Common name	Geography (if known)	Colonises
HilA_1	*Spirometra erinaceieuropaei*	Tape worm	Worldwide/mainly SE Asia	Small mammals, small reptiles and birds
HilA_2	*Pyrenophora tritici-repentis*	Fungus	-	Wheat
	Spirometra erinaceieuropaei	Tape worm	Worldwide/mainly SE Asia	Small mammals, small reptiles and birds
OrgA_1	*Fasciola hepatica*	Common liver fluke	Tropical/Wales	Livers of mammals, sheep and cattle.
OrgA_2	*Bacillus subtilis (pumilus)*	-	-	Soil, hay, wheat, humans
	Cladophialophora psammophila	Black yeast	-	Dead plants, rotten wood, soil
BapA_1	-	-	-	-
BapA_2	-	-	-	-

3.3.4 Generation of LAMP primers

LAMP primers were generated as per methods section 2.4.4. To generate primers within PrimerExplorer V.4, sequences cannot be entered if above 2000 bp, however *bapA* is 11,474bp long. To accommodate the size of the sequence, *bapA* was spilt into 2,000bp sequences in the vicinity of

the PCR primer sets selected in section 3.3.2. To avoid primers that would recognise *Citrobacter* sp. when targeting *bapA*, the first 9,000bp of *bapA* sequence would be considered for LAMP primer design (Table 3.9). The GC content of each gene sequence used was determined; *hilA* and *orgA* being AT rich, *bapA* being GC rich (Table 3.10).

Where possible parameters were maintained as those within methods (section 2.4.4.2) however for *bapA*1, the temperature for F1c/B1c was changed to 60-65°C. This was to allow for the generation of stable primer sets. With *bapA*2, when targeting F2 and B2 around the PCR primer range, F1c and B1c primers could not be generated, so target range was removed, allowing the generation of complete primer sets for this sequence. The sequence length of *orgA* is 600bp, with PCR primers targeting the beginning 200 nucleotides of the sequence. Due to this only 42 primer sets in total could be generated, compared to 1000 sets generated for *hilA*, however viable primer sets that allowed for the generation of loop primers were available, so this did not limit LAMP primer set generation for *orgA*. Overall, when generating the base primer sets for LAMP (4 primers; FIP/BIP, F3/B3) there were minimal changes to parameters required.

When generating loop primers to add to the base LAMP primer set, multiple parameters often needed to be changed to enable loop primer (LP) generation. With *hilA*, *orgA*, and *bapA1*, one base primer set from each could not generate viable loop primers (Table 3.10 & 3.11). To enable LP generation for *hilA*, temperature parameters were expanded to encompass the temperature range for both GC and AT rich sequences, 50-66°C. With *orgA* the GC content range was expanded to encompass that of both GC and AT rich sequences, a GC content of 30-70%, to allow for LP generation. For two base primer sets, both temperature and GC content were expanded before viable LP could be generated, one targeting *orgA* and one targeting *bapA*1. Of the 8 primer sets generated, only 3 required no adjustments to allow for the generation of LP, two targeting *bapA*2 and one targeting *bapA*1.

Table 3.8: The length of gene sequence used to generate LAMP primers as well as the loci of PCR primers on the of the target genes to ensure that primers target the same sequence area where applicable.

Target gene	Sequence reference	Gene sequence split points (bp)	PCR primer set	Starting point of PCR primer product on targeted gene (bp)
hilA	-	Whole sequence used	hilA_1	988
			hilA_2	644
orgA	-	Whole sequence used	orgA_1	138
			orgA_2	80
bapA	*bapA*1	4120 - 6120	bapA_1	5100
	*bapA*2	3100 - 5100	bapA_2	4080

Table 3.9: The GC content of the gene segments used to design LAMP primers and the parameters used within LAMP primer generation as a result.

Gene sequence	GC content (%)	Parameter result	Tm settings (°C)
hilA	43.1	AT rich	55-60
orgA	47.8	AT rich	55-60
*bapA*1	59.1	GC rich	60-65
*bapA*2	58.1	GC rich	60-65

Table 3.10: The number of LAMP primer sets generated within Primer Explorer V4 for each gene targeted, with adapted parameters if applicable, as well as the basic LAMP primer sets chosen for loop primer generation. See appendix 1, for detailed information on each primer set, including base-coordinates for the primers within each target gene.

Sequence targeted	No. primer sets generated	Changed parameters*	Primer sets used in generation of loop primers
hilA	1000	-	22,123,321
orgA	42	-	18,33,42
*bapA*1	1000	F1c/B1c – Tm @ 60-65°C	153, 478, 556
*bapA*2	646	Did not target PCR primer range	43, 439

*parameters were changed to generate primer sets that were stable, complete, or enabled downstream generation of loop primers

Table 3.11: The number of loop primers generated per basic LAMP primer set, as well as those chosen for synthesis of complete LAMP primer sets. See appendix 1, for detailed information on each primer set, including base-coordinates for the primers within each target gene.

Gene target	Basic LAMP Primer set used	Loop sets generated	Modified parameters*	Loop set chosen for synthesis	Primer set reference name
hilA	22	1,712	Tm = 50-66°C	18	hilA2
	123	715	No viable primers	-	-
	321	1254	Tm = 50-66°C	30	hilA1
orgA	18	9	No viable primers	-	-
		63	Tm = 50-66°C GC = 30-70%	19	orgA1
	33	9	No viable primers	-	-
	42	63	GC = 30-70%	23	orgA2
*bapA*1	153	21	Tm = 50-66°C GC = 30-70%	8	bapA1.1
	478	77		25	bapA1.2
	556	21	No viable primers	-	-
*bapA*2	43	40		17	bapA2.1
	439	370		22	bapA2.2

*Parameters were changed to generate viable sets and to avoid fragmented primer sets

Within a LAMP Primer set, a non-specific match was defined as a match found between any forwards primer and any reverse primer. In table 3.12 there were non-specific matches amongst the proposed primer sets, however from these only 1 species, *Mycobacterium* sp. needs to be tested within the lab against 3 primer sets, bapA2.1, orgA1, and orgA2. Other matches were deemed irrelevant to the cattle industry or, when aligned with the non-specific match using BLASTn, did not feasibly produce a product.

Table 3.12: Notable BLASTN Hits observed for prospective LAMP Primer sets. Matches were checked against Primer sets and the target genome: a match and was considered negative if there was no chance of a product being generated (Genome check: as per criteria in section 2.2.3). With advice from Dr. Tim Potter (Westpoint Vets) for relevance within a UK cattle setting, matches that could generate a product with an undesired target were screened for downstream laboratory testing (Lab check), after completion of LAMP methodology optimisation.

Key: N = not further action required, Y = further action required, / = not assessed.

Match	BA1_1	BA1_2	BA2_1	BA2_2	HA1	HA2	OA1	OA2	Notes	Genome check	Lab check
Achromobacter									Found in wet environments, can cause bactermia in immuno-compromised humans	N	/
Aeromonas schubertii									Infects primarily fish, noted in humans. Found in water and sewage, can originate from intestinal sources. Can cause diarrhoea.	N	/
Aggregatibacter									Commensal in human mouths. Associated with periodontitis, actinomycotic lesions and bacterial vaginosis (in humans).	N	/
Angiostrongylus									Parasitic nematode, rats, snails, humans, passed in faeces	N	/
Arthrobacter sp.									Found in soil	N	/
Bordetella sp.									Can infect cattle, dogs, cats, pigs and small ruminants (sheep). Causes respiratory problems	Y	N
Desulfoviobrio sp.									Aquatic however found in Soil, intestine and faeces of cattle (Carli *et al*., 1995)	N	/
Dicrocoelium dentriticum									Liver fluke found in cattle, eggs in faeces	N	/
Echinostoma caproni									intestinal fluke, trematode, molluscs, fish, humans and ruminants	N	/
Leptosphaeria biglobosa									Fungal pathogen of Brassica crops, including canola and rapeseed (which is used as livestock feed - check in UK). Has prevalence in UK	N	/
Mycobacterium sp.									Found in water, causes disease in cattle, ever present in gut?	Y	Y
Paenibacillus sp.									Soil, forage. Rhizobacteria. P. thiaminolyticus has been found to cause disease in ruminants (Jie *et al*., 2008)	N	/
Pseudomonas flourescens									Found in soil near plant roots, can infect humans	N	/
Ralstonia picketti									Found in soil, rivers, lakes. Has been isolated from cattle hide, has been isolated in Ireland	N	/
Rhizobium etti									Fix nitrogen, associated with legumes (common bean)	N	/
Rhodococcus erythropolis									found in soil and water. Used commercially to decontaminate polluted land and waterways. Been noted in cattle, taken from lesions in conjunction with myobacterium.	N	/
Serratia marcescens									Enterobacteriaceae, often found in bathrooms. Pathogen in cattle (Di Guardo *et al*., 1997)	N	/
Spirmometra erinaceieuropaei									Tapeworm infecting humans, cats, dogs, foxes, birds, rodents	N	/
Synechococcus sp.									Prefer well-lit marine and freshwater environ.	N	/
Taenia asiatica									Asian tapeworm, found in humans and pigs. Similar to beef tapeworm but actually only found in pigs	N	/
Thecamonas trahens									Marine in origin	N	/
Triticum aestivum									Wheat - fed to cattle	N	/
Overall matches	6	10	9	3	2	2	3	9			
Matches that need to be tested in lab	0	0	1	0	0	0	1	1			

3.4 Bioinformatics discussion

Conserved genes are present across bacterial genomes of the same species (or genus) and in general evolve slower than the rest of the genome, making them excellent targets for molecular detection (Leekitcharoenphon *et al.,* 2012). Leekitcharoenphon *et al.* (2012) suggested that within *Salmonella* sp., amongst core genes with low variation, there is selection against mutations that can lead to amino acid changes.

Targeting highly conserved genome areas is important in primer development to ensure the efficacy, sensitivity and specificity of the set is maintained. While designing a qPCR assay targeting environmental DNA for bull trout, Wilcox *et al.* (2013) noted the importance of primer specificity, showing that the specificity of their assay was most influenced by base pair mismatches within their primer set. Base pair mismatches near the 3' end of primers have a much larger impact on specificity than at the 5' end (Wilcox *et* al., 2013; Ye *et al*., 2012). The need for specificity holds true for LAMP, as the design of highly sensitive and specific primer set is crucial for LAMP amplification (Parida *et al*., 2008). Parida *et al*. (2008) state that the specificity of LAMP is extremely high as it can amplify a specific gene from a human genome specimen with discrimination down to a single nucleotide difference. Insufficiently specific primers can result in both false positives and false negatives, as well as a reduced efficiency, thus when designing primer sets it is essential to ensure that the target sequence is highly conserved (Wilcox *et* al., 2013; Ye *et al*., 2012). In the case of this study, the target should be areas of highly conserved sequence across multiple *Salmonella* serovars with no nonspecific matches to other organisms and all primer sets should be screen effectively for non-specific matches.

The genome sequences used within this study were collected in Dec 2014; the genomes within the NCBI database may have been updated and differ marginally. At the time of collection, 12 complete *Salmonella* genomes were available on the NCBI database. Of these, 7 genomes were amongst those reported in cattle, *S.* Enteritidis, *S.* Typhimurium, *S.* Dublin and *S.* Newport: with *S*. Dublin host-adapted to cattle. When aligning genomes, resolving power is added by including more than two sequences:

the likelihood of random column identities is lower than that in a pairwise alignment (Stojanovic *et al*., 1999). This made it essential to collect as many complete genomes as possible to increase the accuracy of the alignment. When comparing genomes for homology, good quality DNA sequencing is essential, thus only complete genomes were chosen and partial genomes/contigs were not (Leekitcharoenphon *et al.,* 2012). Complete genomes were collected as initial screening was completed without target genes identified. Ideally a larger amount of *Salmonella* genomes that have been reported to affect cattle would have been included: a complete genome for *S.* Mbandaka would have been ideal, as this was the second most isolated strain from cattle in the UK in 2016 (Lawes and Kidd, 2016). As of March 2017, a complete genome sequence of *S*. Mbandaka (ATCC51958) was made available (https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP019183.1).

Despite the potential discrepancies surrounding evolutionary similarity that Darling *et al.* (2010) highlighted within Mauve, this research is focused on identifying highly conserved sequences across multiple *Salmonella* genomes, thus this is not an issue within the scope of this study. ProgressiveMauve is relatively easy to use and visual clarity of the alignment was useful once familiarity with the program was established. Due to relatively large genome size of *Salmonella* and the seemingly fragmented nature of the genome, when compared to that of *A. baumanni* which is relatively similar in genome size, viewing the entire alignment is confusing. This could be due to seed weight, or potentially, due to the use of 12 genomes, over the 7 used by Wen *et al.* (2014), resulting in an increase of local collinearity. Further investigation into the differences between the amount of LCBs within a species of similar genome size would be interesting but is a tangent to this study.

When observing the complete alignment, a limitation of Mauve was discovered in that the model relies on the genomes used being assembled and annotated correctly. It can be assumed that *S.* Newport (US) and *S.* Enteritidis (2012) located at the bottom of the alignment in Figure 3.2 have been assembled differently to most of the genomes used within the study. Bacterial DNA is circular however, to sequence it, it must be linear and is therefore cut before sequencing. Due to the lack of

an obvious starting point, it may be that *S.* Newport (US) and *S.* Enteritidis (2012) were entered at different starting points and this accounts for the shifts in LCB lines. Incorrectly ordered contigs or differences in genomic alignment can appear as genomic rearrangements in the Mauve viewer and should be researched fully before commented upon as such. A number of genomes are auto-annotated to identify hypothetical genes, only some genomes later undergo full screening for homology to identify the proteins they encode: as seen with *bapA,* a high proportion of hypothetical genes are annotated, which can be at odds with the literature.

As seen in Figure 3.4, *S.* Enteritidis (2012), the genome at the bottom of the alignment, has fragments changing from sense to antisense frequently along the genome. This could be due to how it was sequenced and reassembled, or how it was reported to the NCBI database. For two of the genes chosen for primer development, *S.* Enteritidis (2012) does not have the same arrangement of LCBs as the other 11 genomes, it does however contain the LCBs associated with the genes, but in different positions (Table 3.4a & Table 6a). As *S.* Enteritidis (P1) shows similar alignment of genes chosen for primer development as to the other genomes used, the discrepancies shown by *S.* Enteritidis (2012) were overlooked for the purpose of determining sequence homology. This database entry has subsequently been updated in 2016, after these genomes were collected, with different start points and improved annotation. Future work should look at re-aligning the genomes with this improved sequence.

Whilst Mauve is a useful tool for aligning genomes and identifying conserved sequences, the user interface is not intuitive. Actions such as reordering genomes or zooming into a specific area of sequence are clunky, which makes navigating the alignment slow. Despite this, generation of alignments with Mauve and understanding the visuals, once familiar, was simple and the alignment proved accurate where screened, the LCBs that appeared highly conserved were found to have high specificity across multiple *Salmonella* sp.

S. Dublin was used as the reference genome for the alignment, as this was the most commonly isolated strain from UK cattle from 1998 -2018 (Lawes and Kidd, 2018).

BLAST (Basic Local Alignment Search Tool) is a sequence comparison tool, that aligns the desired sequence against the vast NCBI database to detect similarity which can then be used to conclude homology. Altschul *et al*. (1990) report that BLAST allows for the detection of weak but biologically significant sequence similarities without common DNA sequencing errors, such as replacements or frameshifts, affecting recognition. BLASTN (nucleotide BLAST) was used to align the conserved genes that were identified and confirm their specificity to *Salmonella* sp., as well as determine if the sequences matched organisms other than *Salmonella.*

Hensel (2004) notes that most *Salmonella* Pathogenicity Islands (SPI) have become part of the core genome of *S. enterica*: they code for many of *Salmonella* sp. virulence phenotypes, including host cell invasion and intracellular pathogenesis. Significant structural and functional heterogeneity can be observed within most SPI loci and genes associated with DNA mobility are absent from the majority of SPI, making them areas of genetic stability (Hensel, 2004). Of the 12 different SPI currently described, not all are conserved throughout the *Salmonella* genus, however SPI1 is present on all subspecies and serotypes of *S. enterica* and *S. bongori* that have been analysed so far (Ochman & Groisman, 1996; Hensel *et al.,* 1997). *Salmonella* Pathogenicity Island 1 (SPI1) encodes for a Type III secretory system (T3SS); encoding for the structural components and secreted effector proteins which are required to ensure bacterial uptake in host cells (Altier, 2005; Bajaj *et al.*, 1996; Galan, 1996). Hensel (2004) notes that SPI1 is fixed and stable, making it an excellent target for molecular detection, with genes including two subsets of effector proteins; one set mediates the invasion of nonphagocytic cells through modification of the actin cytoskeleton, the other set with enteropathogenesis.

SPI1 is highly regulated by two regulatory genes, one of which is *hilA* (Mills *et al.*, 1995). A transcriptional activator, *hilA* activates the second transcriptional regulator, *invF,* and regulates the expression of several invasion genes, including *orgA* (Altier, 2005; Galan, 1996; Mills *et al.*, 1995).

Within the Mauve alignment, gene loci associated with *hilA* LCB's were annotated as invasion protein regulator, transcription regulator and in *S*. Typhi was named *iagA,* which is a homolog of *hilA* (Figure 3.4).

Bajaj *et al.* (1996) note that oxygen, osmolarity, pH and the *phoPQ* regulatory system co-ordinately regulate the expression of *hilA*. *PhoPQ* regulates protein and lipid components of *Salmonella* outer membrane in response to innate immune defence (Dalebroux and Miller, 2014). In conditions of low oxygen, high osmolarity, and slight alkalinity, the conditions of the lumen of the ileum where *Salmonella* is reported to initiate infection, *hilA* is upregulated increasing invasiveness by activating the expression of invasion genes, including *orgA* an invasion protein associated with bacterial internalisation (Bajaj, 1996; Murray and Lee, 2000).

Russell *et al.* (2004) note similar when looking at the effect of oxygen on *orgA* specific mRNA copies, using RT-PCR, finding that low oxygen and low osmolarity represses *orgA* expression. As *orgA* is regulated by *hilA* this follows (Jones and Falkow, 1994). *orgA* is coded within SPI1 and is involved in invasion and secretion, it has been shown that polar insertions into the *orgA* sequence prevents the formation of the type III secretion needle structure which plays an important role in *Salmonella* pathogenicity (Klein *et al*., 2000; Kubori *et al.*, 1998). Within the Mauve alignment gene loci associated with *orgA* LCBs were annotated as Type III secretion apparatus protein, cell invasion protein, oxygen-related invasion protein and needle complex assembly protein (Figure 3.5), which concurs with the literature (Klein *et al*., 2000; Kubori *et al.*, 1998).

Latasa *et al*. (2005) demonstrated that a protein encoded by the locus 'stm2689' was required for biofilm formation, renaming the locus *bapA*, due to product homology with Bap produced by *Staphylococcus aureus*. Donlan (2002) states that biofilms are a community of microbes adhered to a surface enclosed within a matrix that consists primarily of polysaccharides. Activation by CsgD promotes the co-ordinated expression of BapA, cellulose, and fimbriae, which comprise the

Salmonella matrix of the biofilm. Latasa *et al*. (2005) suggest that BapA may promote cell-cell interactions, acting as a receptor and ligand between two bacterial clusters.

A study by Biswas *et al.* (2010) noted that the *bapA* gene can be targeted for the genus-specific detection of *Salmonella* sp. A set of PCR primers were tested against 34 different *Salmonella* serotypes for the presence of biofilm-associated protein (*bapA)*, all 67 isolates yielded positive results. Biswas *et al.* (2010) noted that *bapA* is referred to as 'stm2689' in literature and this annotation was seen in the *S.* Typhimurium (LT2) gene loci associated with *bapA* LCB. Within the Mauve alignment the gene loci associated with *bapA* LCBs were annotated as VCBS repeat containing protein, hypothetical protein, large repetitive protein and pseudogene (unknown/true/frameshift, proline/threonine rich) (Table 3.6). *bapA i*s the second largest open reading frame within *Salmonella*, which may account for why it was originally a hypothetical repeat containing protein (Latasa *et al*., 2005). Within *S.* Typhi (CT) the locus code for *bapA* is STY2875, which Latasa *et al*. (2005) found to be a homolog of *bapA*, which is located on the *S.* Typhi pathogenicity island SPI-9: within other *Salmonella* sp. *bapA* is not currently associated with a pathogenicity island. After alignment in BLAST, it was noted that the *bapA* sequence matches to that of *Citrobacter* sp. after 9,000 bp until the end of the gene, because of this when targeting *bapA* for primer development only the first 9,000bp were used.

It can be seen with well researched genes, such as *hilA*, that the gene name or protein function is correctly annotated, within the NCBI database. However, *bapA* was first described recently, resulting in the annotation of genomes largely reporting *bapA* as a hypothetical gene or pseudogene.

PCR is common within molecular microbiology and pathology labs: it is a sensitive and specific nucleic acid technique (Jadidi *et al*., 2012; Hadjinicolaou *et al*., 2009; Farrell *et al*., 2005; Pathmanathan *et al*., 2003). *Salmonella* sp. have previously been detected by multiple studies using PCR, Pathmanathan *et al* (2003) found that *hilA* gene-targeting PCR primers were specific for *Salmonella* sp. and noted that PCR is a well-documented and established technique for diagnosing infections. To determine whether the genes identified as highly specific by Mauve can be used to develop primers for the detection of

Salmonella sp. PCR primers were generated technique (Jadidi *et al*., 2012; Hadjinicolaou *et al*., 2009; Farrell *et al*., 2005; Pathmanathan *et al*., 2003).

Primer-BLAST was used to generate 5 PCR primer sets per *Salmonella*-specific gene, 15 sets in total. Ye *et al.* (2012) state that Primer-BLAST is a general-purpose target-specific PCR primer design tool, that is flexible, highly sensitive and easy to use, with results showing the potential for any unintended targets. Using BLASTN, primer sets were assessed and 2 sets per gene were chosen for production. Non-specific matches were assessed for relevance to the cattle industry to ensure that the sequences targeted by the PCR primers could be adapted for LAMP primer generation (Figure 3.13).

No undesired matches were seen in either *bapA* PCR set. In both *hilA* sets S*pirometra erinaceieuropaei* was found as a match. *Spirometra erinaceieuropaei* is a tapeworm that can be found worldwide and affects small mammals, reptiles and birds. Within the hilA_2 PCR set a match to *Pyrenophora tritici-repentis*, a fungus can colonise wheat, was seen. *Fasciola hepatica*, the common liver fluke, which has been seen in sheep and cattle in Wales was matched within the orgA_1 set. *Bacillus subtilis* was matched within the orgA_2 primer set and is ubiquitous with the environment. Despite potentially problematic matches, the PCR primer sets within this study were to determine whether the target genes are pan-*Salmonellae* only, thus matches here were taken into consideration when developing LAMP primers (Figure 3.13).

LAMP is simple, cost-effective, easy and rapid nucleic acid detection technique; after mixing, amplification and detection of the sample can be carried out in a single step (Notomi *et al*., 2000; Parida *et al.,* 2008). LAMP is noted for its high specificity and high amplification efficiency, as well its simplicity, due to the isothermal nature of the assay (Okamura *et al*., 2008; Parida *et al*., 2008).

PrimerExplorer is a LAMP primer designing software, developed by Eiken alongside Notomi *et al*. (2000), thus this software is presented by those who developed the LAMP technique. For each gene targeted, 2 complete sets of LAMP primers, including loop primers, was generated: six sets in total. Multiple LAMP primer sets were generated, however two for each gene were selected for loop primer

development: for *hilA, orgA,* and *bapA*1, if a chosen set did not produce viable loop primers, a third primer set was selected (Tables 3.16 and 3.17). For *bapA*2, when targeting F2 and B2 around the PCR primer range in the middle of the 2000bp sequence, F1c and B1c primers could not be generated, and thus this targeting range was removed allowing the generation of primer sets. Ideally future LAMP primer designing software would allow for the generation of the base LAMP primer set and accompanying loop primer set simultaneously under the same parameters to save time and ensure for continuality across the complete LAMP primer set.

Notomi *et al.* (2000) noted that the best results for LAMP could be obtained with target DNA being between 130-200 bases in length. Therefore, the size of the target DNA should be set to less than 300bp, including B3 and F3. As seen in Appendix 1, all LAMP primer sets used downstream target areas of DNA less than 250bp, thus should result in optimum amplification.

When using PrimerExplorer V.4 (PE V.4, https://primerexplorer.jp/e/) there were several limitations found. Whilst only being able to enter 2,000bp is not an issue for smaller genes, it meant that in the case of *bapA* that there potentially could have been a part of the 9,000bp sequence that would have produced primer sets that were stronger and more stable than those produced within this study. PE V.4 will only work in certain internet browsers and an out-dated version of JAVA is needed for the software to run. When a sequence is entered into PE V.4, this sequence is remembered until the entire browser is closed and re-opened, limiting research to one sequence at a time. Additionally, PE V.4 will generate primer sets that are unstable or incomplete and present them to the user, rather than automatically screening them out. When generating primer sets, PE V.4 will generate a maximum of 1,000 primer sets, however only 100 can ever be viewed, there is no option to see all the primer sets generated. This, combined with the incomplete or unsuitable sets, limits the number of viable primers sets generated. The generation of LAMP primer sets that do not allow the generation of matching loop primers further limits the primer sets you can choose from. As seen in methods section 3.2.3, the generation of the F3/B3 and the FIP/BIP primers is separate to the generation of the loop primers.

Whilst the LAMP assay can be used without loop primers, the speed of the assay is greatly improved with loop primers, as they increase the number of starting points for DNA synthesis to occur. Overall, whilst PE V.4 is free, and from those who developed the assay, it is recommended that different software, if available, is used for the development of LAMP primers due to the issues encountered within this study.

As LAMP requires 6 primers, as opposed to the 2 required in PCR, specificity screening was more in-depth. Each primer within a set was aligned using BLASTN, the results were collected and collated within a table and manually compared for matches between forward primers and backward primers (See Appendix 2 for an example). Due to the nature of nucleic amplification, matches between two forward primers or two backward primers were deemed unlikely to generate a product. As the LAMP primers were to be used for in-situ testing, the nonspecific matches were checked thoroughly, by use of relevant literature and under veterinary consultation, as well as by determining sequence positioning on the matched genome. For example, within hilA2, matches were seen for *E*. *coli,* a common gastrointestinal bacterium in cattle, however the F3 match was on a plasmid, whilst the BIP and B3 matches were on the genome. Due to locality, the set would be unlikely to generate a product in the presence of *E. coli* and thus can be ruled out as a match. *Mycobacterium* sp. was found to potentially generate product within LAMP primer sets bapA2.1, orgA1, and orgA2, and therefore should undergo testing under laboratory conditions to determine whether the signal produced is strong enough to affect the detection of *Salmonella* sp. (Figure 3.18).

Future work which could benefit this study would be an alignment containing additional complete *Salmonella* genomes, including that of *S.* Mbandaka, to increase the specificity of genes targeted by primer sets. This could be achieved by sequencing and assembling the genomes, or by searching multiple genomic databases, however most published genomes are reported to the NCBI.

When generating primers to allow for the detection of an organism, ensuring that the primers are highly specific and target a conserved sequence is important. For detecting conserved sequences

across multiple genomes, Mauve is a useful tool with clear visuals. BLASTN is irreplaceable when determining the specificity of a sequence, due to the expansive database it can align nucleotides to. Generating PCR primers with Primer-BLAST was simple, in contrast to designing LAMP primers using PrimerExplorer V4. Whilst a LAMP primer set is innately more complicated to generate than that of a PCR set, just due to the increase in primer number, most difficultly was due to the interface and complex requirements of the PrimerExplorer software and thus is off-putting to those wishing to utilise loop-mediated amplification. Overall, three highly conserved and pan-*Salmonella* specific genes have been identified and targeted for both PCR and LAMP primer development. Six PCR primer sets were generated, and 8 LAMP primers sets complete with loop primers were generated, all of which were tested downstream within this research.

4. Polymerase chain reaction (PCR)

4.1 PCR introduction

Polymerase Chain Reaction (PCR) is a sensitive and specific nucleic acid amplification technique that uses heat denaturation of double-stranded DNA to promote the next round of DNA synthesis (Farrell *et al.*, 2005; Nagamine *et al.*, 2002). Pauda *et al*. (1999) noted that PCR revolutionised molecular research and is currently the most widely used nucleic acid amplification technique for the diagnosis of infectious diseases (Parida *et al*., 2008; Nagamine *et al.*, 2002). Commonplace in molecular microbiology and molecular pathology labs, PCR has increased the speed and sensitivity of infectious disease detection and is often used to detect fastidious microbes that cannot be easily cultivated (Farrell *et al.*, 2005; Stone *et al*., 1998).

The speed of molecular testing allows for the early implementation of specific and effective therapy, potential to improve outcomes and decreased drug-related selection pressure and costs (Francois *et al*., 2011; Mobed *et al*, 2019). Murphy and Bustin (2009), state that the combination of molecular diagnostics with therapeutics is a key component of integrated healthcare.

In order to visualise PCR products, gel electrophoresis is commonly used. DNA fragments migrate through the gel towards the anode, due to the negatively charged phosphate backbone of DNA (Voytas, 2001). As this migration is limited by the frictional force imposed by the gel matrix, larger DNA fragments have a slower migration than smaller fragments, resulting in the separation by size (Voytas, 2001). By utilising known standards in the form of a molecular weight marker (DNA ladder), DNA fragment size can be calculated (Voytas, 2001).

Conventional PCR methods require laboratory space and are considered time-consuming, labour-intensive, complex, and expensive (Mobed *et al*., 2019; Verdoodt *et al*., 2017; Cheung and Kam, 2012; Parida *et al*., 2008; Kaneko *et al*., 2006; Mori *et al*., 2001; Notomi *et al*., 2000). Despite these disadvantages, the sensitivity and specificity of PCR has resulted in the method being considered the gold-standard of molecular detection and numerous PCR methods detecting different *Salmonella* genes have been developed (Moore and Feist, 2007; Farrell *et al.*, 2005). Stone *et al*. (1998) noted that

in asymptomatic animals, low levels of *Salmonella* in samples is common, suggesting PCR as a useful tool in these cases where the aetiological agent is not determined by culture.

This study aims to use PCR to confirm that the genes, *bapA, hilA,* and *orgA*, which were identified using bioinformatics techniques as pan-*Salmonella* specific are indeed specific, and thus can be used as targets for loop-mediated isothermal amplification (LAMP) based detection assays. To achieve this, the PCR primer sets were tested against multiple *Salmonella* serovars.

4.2 PCR methodology

Genomic DNA was isolated for the *Salmonella* sp. strains and *E. coli* control listed in section 2.1.3 using the methodology outlined in section 2.3.1. Following quantitative and qualitative analysis of the DNA, PCR was performed according to section 2.5.2., unless otherwise stated. All PCR products were visualised by gel electrophoresis, as described in section 2.3.2.

4.2.1 Optimising the HotStarTaq PCR methodology

To troubleshoot annealing temperature, the method used in 2.5.2 was adapted as shown in Table 4.1. Differing levels of primer volume were used to increase primer concentration from the original 2 µM (1µl), to 2.5µM (1.25µl), to 3µM (1.5µl), as seen in Table 4.2. *S.* Dublin was used as template DNA unless stated otherwise.

Table 4.1: The cycling conditions used to determine the optimum annealing temperature for each PCR primer set.

Stage	Temperature (°C)	Time (mins)	Cycles
Initial Denaturation	95	5	1
Denaturation	94	0.67	30
Annealing	50-60*	0.67	
Extension	72	1	
Final extension	72	10	1

*Annealing temperatures were varied by 2°C increments within the stated range.

Table 4.2: HotstarTaq PCR reaction mixture for optimising for annealing temperature

Reagent	Volume (µl)
HotStarTaq	10
Forward primer	1-1.5
Backwards primer	1-1.5
Template DNA	0.55 – 3*
Nuclease free water	5 – 7.45**
Total volume (µl)	**20**

*concentrations vary due to concentration differences in Template DNA.

**Concentration of template DNA in overall mix 100ng. Overall volume made up to 20µl with Nuclease free water.

4.2.2 Determining primer specificity

To determine how specific the primer sets were to a multitude of *Salmonella* serovars, primers were tested with the genomic DNA of 6 *Salmonella* strains (*S.* Bovismorbificans, *S.* Dublin, *S.* Enteritidis, *S.* Montevideo, *S.* Newport, *S.* Typhimurium) using the method in section 4.2.1 was used, with optimum annealing temperature and primer concentration (3µM in final reaction volume) for each primer set, see Table 4.3.

Table 4.3: The optimal annealing temperature and primer volume per PCR primer set.

PCR Primer set	Annealing temperature (°C)	Primer volume (µl)
bapA_1	58	1.5
bapA_2	58	1.5
hilA_1	54	1.5
hilA_2	54	1.5
orgA_1	56	1.5
orgA_2	54	1.5

4.3 Results of PCR for the detection of *Salmonella* sp.

4.3.1 PCR assays using the Dream *Taq* Green master mix

When using the Dream *Taq* methodology, section 2.4.1., all PCR primer sets (Table 2.4) were tested against *S.* Dublin, however no products were seen, despite visible clear ladders, however brightness was seen in wells.

4.3.2 PCR assays using the HotStarTaq Plus Master mix

When using the HotStarTaq Plus methodology, section 2.5.2, initially hilA_1, targeting the *hilA* gene, was used against a panel of *Salmonella* sp. (*S.* Bovismorbificans, *S.* Dublin, *S.* Enteritidis, *S.* Montevideo, *S.* Newport, *S.* Typhimurium; Figure 4.1). Faint products were seen for *S.* Dublin and *S.* Enteritidis, 2 out of 6 strains, the expected product length was 660bp. In Figure 4.1, faint products can be seen mobility near 600bp fragment in the marker lane for *S.* Dublin and *S.* Enteritidis, the bands produced are slightly lower than expected for hilA_1.

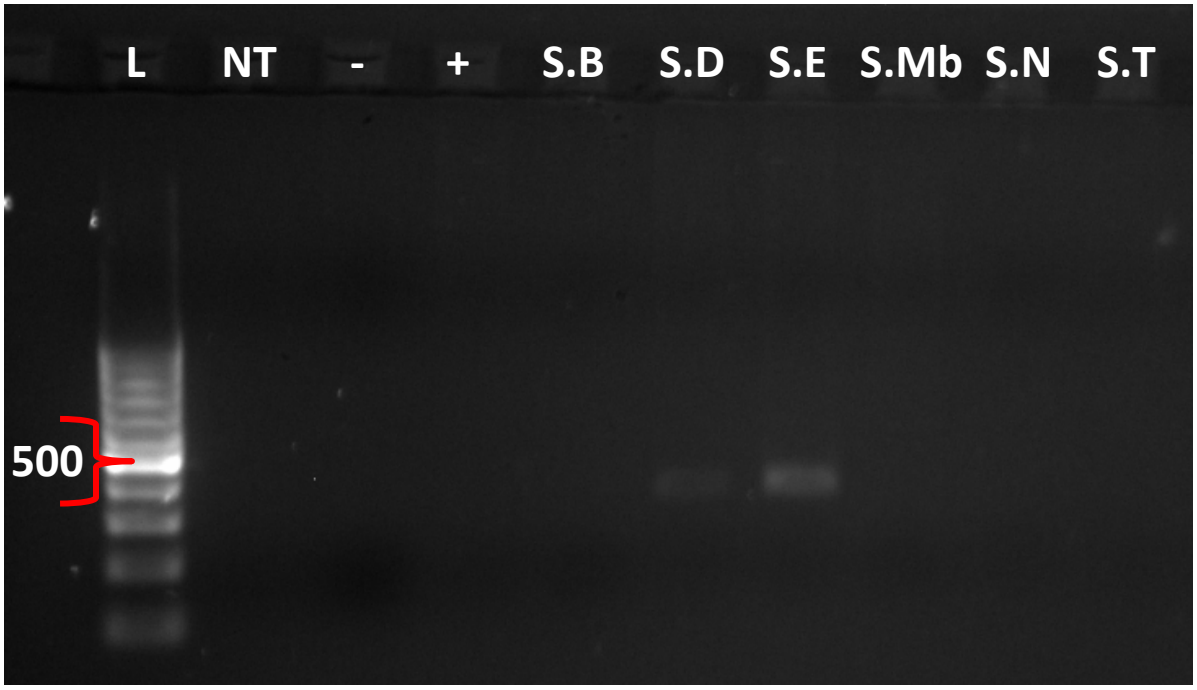

Figure 4.1: Gel showing the results of HotStarTaq PCR for the detection of *Salmonella* sp. using the hilA_1 primer set at 65°C

Key: L= DNA marker ladder, NT = No template DNA, - = negative control, + = positive control, S.B = *S.* Bovismorbificans, S.D = *S.* Dublin, S.E = *S.* Enteritidis, S.Mb = *S.* Mbandaka, S.N = *S.* Newport, S.T = *S.* Typhimurium, 500 = 500bp marker in the ladder

4.3.3 Optimising the HotStarTaq PCR methodology

To improve upon the PCR amplifications seen in Figure 4.1, a range of annealing temperatures and primer volumes were tested, as per method in section 4.2.1., with *S.* Dublin genomic DNA as the template

For orgA_1, based on band brightness, maximum amplification was achieved with 2µM primer concentration and an annealing temperature at 60°C, as well as with 3µM primer volume at 56°C (Figure 4.2). For the remaining primer sets, maximum amplification was achieved at a primer concentration of 3µM (Figures 4.3 – 4.7). Maximum amplification was observed at 52-54°C for orgA_2, and for bapA_1 and bapA_2 was seen at 58°C (Figures 4.3, 4.6, 4.7). For hilA_1 and hilA_2, maximum amplification was seen at 54°C (Figures 4.4, 4.5).

For orgA_1 and orgA_2, product length should be at 384bp and 437bp respectively. Bands for orgA_1 sit just below the 400bp marker (figure 4.2) and those for orgA_2 sit at the 400bp mark (figure 4.3). Product length should be at 660bp for hilA_1 and 344bp for hilA_2. Whilst bands for hilA_2 regularly sit between the 300bp and 400bp markers (figure 4.4), product bands for hilA_1 sit at or just above 700bp (Figure 4.5). In figure 4.6 the bands of product for bapA_1 sit just above the 400bp mark: bapA_1 product length should be at 425bp. For bapA_2, bands sit below the 600bp marker, product length for bapA_2 should be 591bp (figure 4.7).

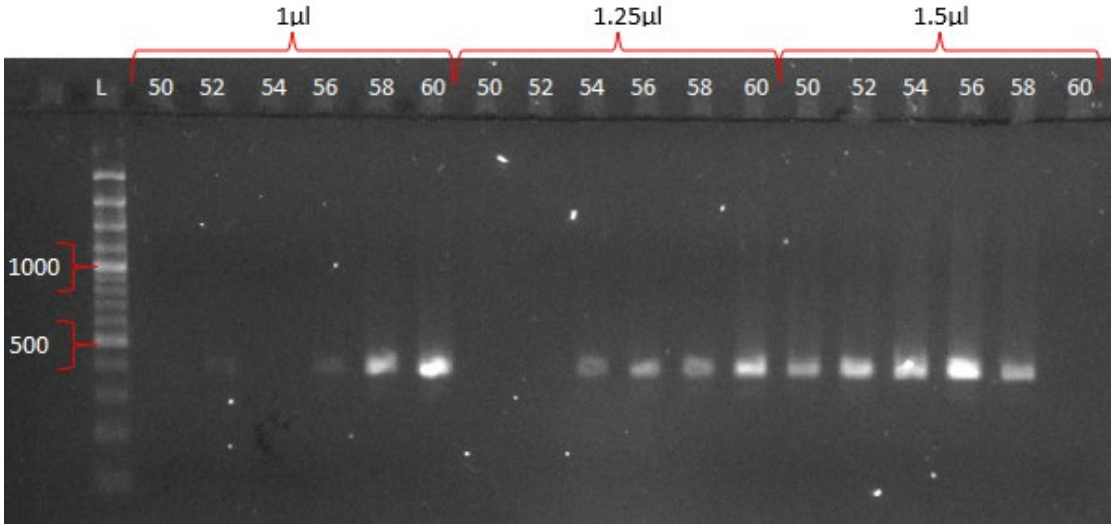

Figure 4.2: A gel showing the effect different concentrations of the orgA_1 primer set have at different annealing temperatures against *S.* Dublin.

Key: 1-1.5µl = primer concentration, L = DNA ladder, 50-60 = annealing temperature (°C), 500 = 500bp marker in the ladder

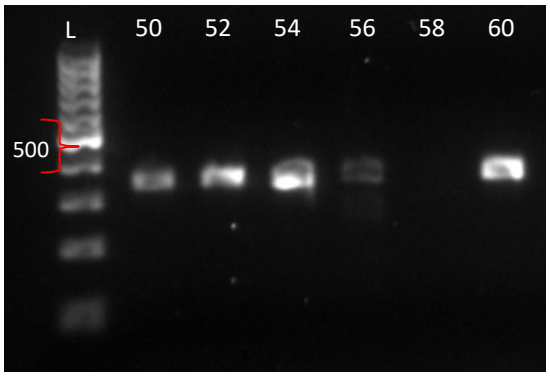

Figure 4.3: Gel electrophoresis showing differing annealing temperatures for the amplification of *S.* Dublin using 1.5µl of orgA_2 primer set.

Key: L = DNA ladder, 50-60 = annealing temperature (°C), 500 = 500bp marker in the ladder

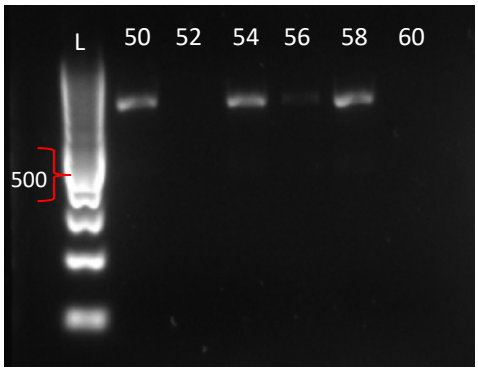

Figure 4.4: Gel electrophoresis showing differing annealing temperatures for the amplification of *S.* Dublin using 1.5µl of hilA_1 primer set.

Key: L = DNA ladder, 50-60 = annealing temperature (°C), 500 = 500bp marker in the ladder

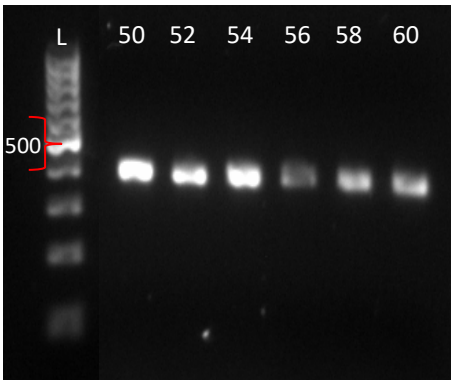

Figure 4.5: Gel electrophoresis showing differing annealing temperatures for the amplification of *S.* Dublin using 1.5µl of hilA_2 primer set.

Key: L = DNA ladder, 50-60 = annealing temperature (°C), 500 = 500bp marker in the ladder

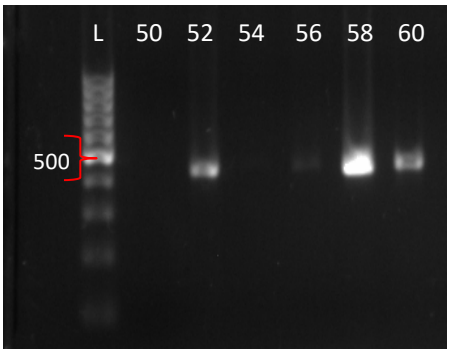

Figure 4.6: Gel electrophoresis showing differing annealing temperatures for the amplification of *S.* Dublin using 1.5µl of bapA_1 primer set.

Key: L = DNA ladder, 50-60 = annealing temperature (°C), 500 = 500bp marker in the ladder

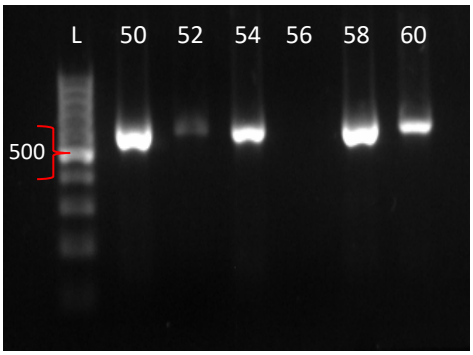

Figure 4.7: Gel electrophoresis showing differing annealing temperatures for the amplification of *S.* Dublin using 1.5µl of bapA_2 primer set.

Key: L = DNA ladder, 50-60 = annealing temperature (°C), 500 = 500bp marker in the ladder

4.3.4 Determining primer specificity

To ensure that the PCR primers designed were able to detect multiple *Salmonella* serovars, the primer sets were tested against genomic DNA from *Salmonella* sp., as per the methodology in section 4.2.2, with *E. coli* as a negative control. All primer sets detected *S.* Dublin, with orgA_1 detecting all *Salmonella* serovars tested (Table 4.4, Figure 4.8). *S.* Bovismorbificans was the least detected serovar, only detected by hilA_2 and orgA_1 (Table 4.4). Most primer sets detected 4 out of 6 *Salmonella* serovars tested, accept bapA_1, which only detected *S.* Dublin and *S.* Typhimurium (Table 4.4).

Table 4.4: The specificity of the PCR primer sets, using the optimal primer volume and annealing temperature for each set when targeting *S.* Dublin (Table 4.3), using HotStarTaq PCR method against various *Salmonella* serovars.

Key: X = no product seen after gel electrophoresis, D = product seen after gel electrophoresis

Primer set	Type of Bacterial Genomic DNA Tested						
	E. coli	*S.* Bovismorbificans	*S.* Dublin	*S.* Enteritidis	*S.* Montevideo	*S.* Newport	*S.* Typhimurium
bapA_1	X	X	D	X	X	X	D
bapA_2	X	X	D	D	D	D	X
hilA_1	X	X	D	D	D	D	D
hilA_2	X	D	D	D	D	D	X
orgA_1	X	D	D	D	D	D	D
orgA_2	X	X	D	D	D	X	D

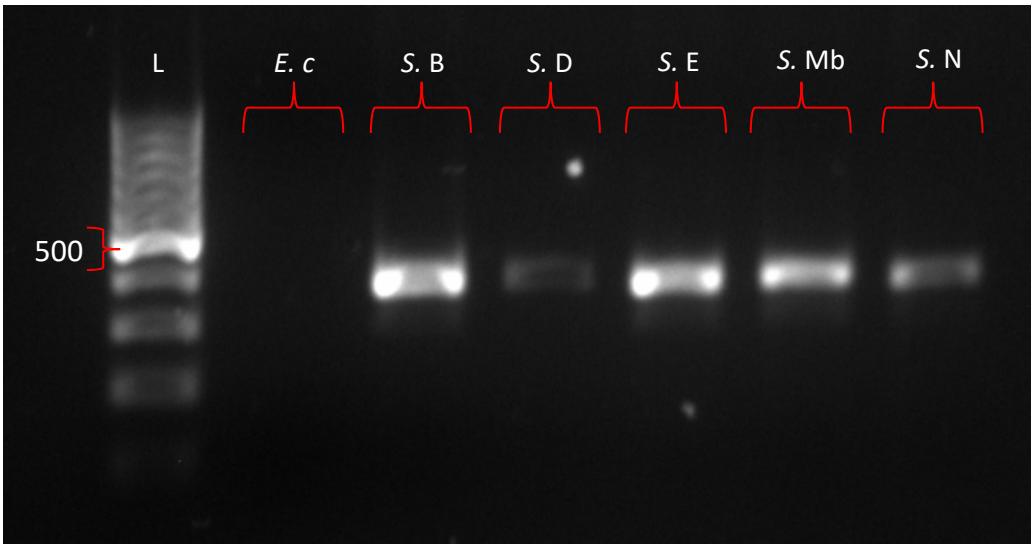

Figure 4.8: The amplification results of optimised HotStarTaq PCR method for orgA1 primer set against a selection of *Salmonella* serovars.

Key: L = DNA ladder, 500 = 500bp marker in the ladder, *E. c* = *E. coli*, *S.* B = *S.* Bovismorbificans, *S.* D = *S.* Dublin, *S.* E = *S.* Enteritidis, *S.* Mb = *S.* Mbandaka, *S.* N = *S.* Newport

4.4 PCR Discussion

PCR amplification is a powerful tool in microbiological diagnostics, widely used in infectious disease diagnostics and molecular research (Parida *et al*., 2008; Malorny *et al*., 2003); Nagamine *et al.*, 2002; Pauda *et al*., 1999). Due to high sensitivity and specificity, PCR is considered the gold-standard of nucleic amplification techniques, a benchmark that newly developed techniques need to reach. Within this research PCR was used to show that genes identified by bioinformatic techniques as candidate pan- *Salmonella* markers could be used as effective targets for nucleic amplification.

When using the Dream *Taq* Green mastermix, PCR did not produce any products, despite clear ladders. This was potentially due to the formation of primer dimers; brightness was observed within wells suggesting too much primer was added during the reaction set up. Whilst the PCR primers were designed to not cross-react within a set, non-specific amplification at room temperature can result in primer dimers. By using a *Taq* with hot start activity, the potential for primer dimers is reduced, as the *Taq* is not active until initial activation step denaturation. Thus, HotStarTaq PCR master mix was used. When switching between master mixes, the following changes in protocol were made; overall reaction volume was reduced from 50µl to 20µl, annealing temperature was lowered from 65°C to 55°C. Whilst the volume of primer added was reduced from 2µl to 1µl, the overall concentration remained at 2µM.

As shown in Figure 4.1, faint product bands could be seen showing hilA_1 primer set could detect *S.* Enteritidis and *S.* Dublin. However due to the faintness of the bands, it was concluded that the HotStarTaq protocol required further optimisation.

Within the cycling protocol, it was noted that the annealing temperature of 65°C was high. Within thermal cycling, when an annealing temperature is too high primers will not bind to template DNA. When annealing temperature is too low, the formation of non-specific amplification or secondary structures can form, slowing or halting amplification. Thus, correct annealing temperature is required to allow to maximum amplification. This is often 5°C lower than the primer melting temperature. The

melting temperature accompanying the custom oligonucleotides sent by Sigma-Aldrich was 65°C, therefore annealing temperature should be at ~60°C. However, when designing the PCR primers used within this research, the melting temperature set by PrimerBLAST was 60°C, thus the annealing temperature should be ~55°C (Table 3.12). The melting temperature of primers can be affected by salt concentration, which could explain the difference in melting temperature recommendations. To optimise the HotStarTaq PCR methodology, different annealing temperatures and primer concentration were used, to generate a greater product yield to enable maximum product yield.

As seen in table 4.3, a primer concentration of 3µM, was determined to produce maximum amplification within the HotStarTaq protocol, an increase of 1µM per reaction. Whilst orgA_1 had good amplification at 2µM, at 60°C, a universal concentration across primer sets was preferred, to allow for a consistent protocol. Across the primer sets, maximum amplification was observed at 54-58°C annealing temperature, around the annealing temperature suggested by PrimerBLAST and lower than that suggested by Sigma-Aldrich (Table 4.3). It is recommended that when developing a novel PCR protocol, annealing temperature is determined experimentally to improve PCR amplification and allow for clearer results.

The product bands within the gels are largely within the expected bp lengths when compared to the ladder (Table 3.12). For hilA_1 the expected product size was 660bp, however bands often sat at or just above the 700bp ladder marker (Figure 4.5). This is thought to be due to the small size of the gel combined with a quick run time which resulted in a compressed ladder. To allow rapid observation of PCR reaction outcomes, the agarose gels were run at higher voltage to achieve fast resolution, therefore only an approximate product size could be determined. To avoid ladder compression, agarose gels could be run for an extended period, at a lower voltage, with one set of wells per gel cast. This would also allow for analysis and quantification of the product bands seen within the agarose gels. Additionally, future work could look at confirmation of the products generated via the PCR primer sets by sequencing.

When tested against a panel of *Salmonella* serovars, all primer sets detected *S*. Dublin genomic DNA and none detected the negative control, *E. coli* - a common gastrointestinal bacterium (Table 4.4). Primer set orgA_1 detected all 6 salmonellae strains tested, whilst bapA_1 only detect 2/6 strains tested (table 4.4). *S.* Bovismorbificans was the least detected serovar only detected by hilA_2 and orgA_1 (Table 4.4). Several steps could be taken to improve amplification, further optimisation such a increasing template concentration, increasing the amount of amplification cycles, or re-designing primers to improve specificity. When designing primers using PrimerBLAST, the sequences for target genes were sourced from *S.* Typhimurium (LT2): base pair differences at the PCR primer target site could reduce primer efficacy, reducing amplification. However, as PCR was used as a determination of whether the genes targeted could be used in primer design for *Salmonella* detection, further optimisation was deemed outside the scope of this research.

It has been noted that the complex nature of PCR is a disadvantage, sensitive to minute changes in protocol, equipment calibration and sample preparation, with limitations including low amplification efficiency and low sensitivity (Verdoodt *et al*., 2017; Mori *et al*., 2001; Notomi *et al*., 2000). Due to thermal cycling, DNA extraction and post-PCR analysis, the methodology of PCR is considered elaborate and time-consuming (Cheung and Kam, 2012; Francois *et al.*, 2011; Parida et al., 2008; Nagamine *et al.*, 2002; Mori *et al*., 2001; Notomi et al., 2000, Fredricks and Relman, 1998). Notomi *et al*. (2000) noted that PCR protocols are often cumbersome to adapt to routine use, particularly in peripheral or private health care settings.

PCR is sensitive to a variety of inhibitors, which need to be removed before amplification of the target DNA (Silva *et al*. 2011). Including reagents commonly used in research, Al-soud and Radstrom (1998) found that blood and homogenates of cheese, meat and faeces inhibited PCR amplification. DNA extraction can compromise sensitivity, due to recovery loss, and requires specific equipment, qualified personnel, and is considered time-consuming and laborious (Vinayaka *et al*., 2018; Kaneko *et al*., 2006).

PCR is considered an expensive method due to the need for skilled professionals as well as high precision equipment and the cost of commercial PCR and DNA extraction kits (Mobed *et al*., 2019; Verdoodt *et al*., 2017; Parida *et al*., 2008; Kaneko *et al*., 2006; Mori *et al*., 2001; Notomi *et al*., 2000). Silva *et al*. (2011) noted that the high cost of commercial DNA purification kits make routine use difficult, especially in developing countries (Francois *et al*., 2011).

However, is should be noted that efforts have been made to streamline PCR. Pathmanathan *et al.* (2003) developed a PCR protocol that can detect *Salmonella* sp. in under 18hrs by using human faecal samples directly without extracting genomic DNA removing lengthy DNA extraction. Whilst they noted that the sensitivity was reduced by normal flora and inhibitors in the stool sample, they utilised a 10- and 20-fold dilution method which improved amplification.

Quantitative PCR (qPCR) monitors the amplification of target DNA as it occurs, through the use of a fluorescent dye or reporter, shortening protocol time by removing post-amplification analysis via gel electrophoresis, as well as allowing for both qualitative and quantitative target measurement (Cheung and Kam, 2012; Lee *et al.*, 2009). Malorny *et al*. (2004) noted that the use of qPCR is increasing for the detection of samples contaminated with *Salmonella* within the food production chain, as it allows for specific, rapid, and reliable detection. When comparing conventional PCR to qPCR, qPCR has several advantages, including faster cycling; closed reaction tubes; potential for use of specific probes; and immediate detection of results, eliminating post-amplification electrophoresis and limiting contamination issues faced with conventional PCR (Moore and Feist, 2007; Pauda *et al.*, 1999).

Farrell *et al*. (2005) present a qPCR assay for *Salmonella* sp. detection from stool cultures as accurate as conventional methods but considerably faster. They note that *Salmonella* detection through faecal matter is difficult: DNA extraction was still needed before nucleic amplification could occur. Farrell *et al*. (2005) noted that the pan-*Salmonella* assay was considerably less sensitive with DNA extracts from stool samples compared with colony lysates.

When compared to conventional PCR, qPCR is often simplified whilst retaining the same level of sensitivity and specificity. Parida *et al.* (2008) noted qPCR has the ability for high throughput and is relatively easy to platform, however the machines required are still expensive, require additional lab-based equipment and specialised operators. Due to this, Parida *et al.* (2008) suggest that qPCR is economically unfeasible and therefore unlikely to be widespread in clinics and developing countries (Francois *et al*., 2011).

In conclusion, development of a PCR assay was as laborious and time-consuming as previously suggested, however once the assay was optimised, was reliable. Generating PCR primers is relatively simple, thus this study used PCR as proof of concept to ensure gene targets, in this case *bapA, hilA,* and *orgA*, were pan-*Salmonella* before downstream use in the design of LAMP primers and LAMP assay. Due to this, the sensitivity and robustness of the PCR assay was not studied, which future work could explore. Whilst PCR is a useful tool in infectious disease research and diagnostics, it is not simple, robust, or cheap enough to develop for pen-side use.

5. Loop mediated isothermal amplification (LAMP)

5.1 LAMP Introduction

To enable point of care detection and targeted treatment, the development of robust and rapid diagnostic tests are needed to improve animal welfare, limit loss of product, and help promote antibiotic stewardship. Bovine salmonellosis can result in the death of calves within 48hours of infection: *S.* Dublin infections in newly and persistently infected herds lead to unacceptable levels of morbidity and mortality (Nielsen, 2013). To control the spread of infection, Lomborg *et al.* (2007) noted that it is important to have efficient, cost effective, and reliable diagnostic tools for the detection of persistently infected animals to control the spread of infection within and between herds effectively.

Loop-mediated isothermal AMPlification (LAMP) is a nucleic acid amplification technique that uses six primers specifically designed to recognise eight distinct regions on the target gene (Figure 5.1): the assay proceeds at a constant temperature using DNA polymerase with strand displacement activity, commonly *Bst* polymerase, to amplify the target DNA (Parida *et al*., 2008). LAMP is reported as both highly specific and highly efficient in amplification, as well as cost-effective (Mori *et al.,* 2001). Additionally, the LAMP assay is simple to use, rapid and allows for ease of detection (Francois *et al.*, 2011; Notomi *et al.,* 2000; Parida *et al*., 2008).

During LAMP assays, initially a non-cyclic step occurs, where the primers schematically presented in Figure 5.1 recognise and associate with the target DNA (Figure 5.2). DNA polymerase, with strand displacement activity, binds where the primers have associated and begins synthesising DNA complementary to the target DNA starting at the 3' end of the F2 region of the FIP (Parida *et al*., 2008). Outside of the FIP, the F3 primer anneals to the F3c region on the target DNA and initiates strand displacement DNA synthesis. The DNA strand synthesised from the F3 primer forms a double strand with the template DNA strand (Parida *et al*., 2008). This displaces the FIP-linked complementary strand, releasing it as a single strand, which forms a stem-loop structure at the 5' end due to complementary F1c and F1 regions. The single strand in the middle of the stem-loop structure acts as

a template for DNA synthesis initiated by BIP (Parida *et al*., 2008). BIP anneals to the DNA strand and synthesis of complementary DNA occurs from the 3'end: through this the DNA reverts from a loop structure into a linear structure. The B3 primer anneals to the outside of BIP, displacing it, and DNA is synthesised from the 3'end of the B3 primer. The displaced BIP-linked complementary strand forms a structure with stem-loops at each end, formation of this 'dumbbell' structure initiates the exponential amplification phase (Figure 5.3; Parida *et al*., 2008).

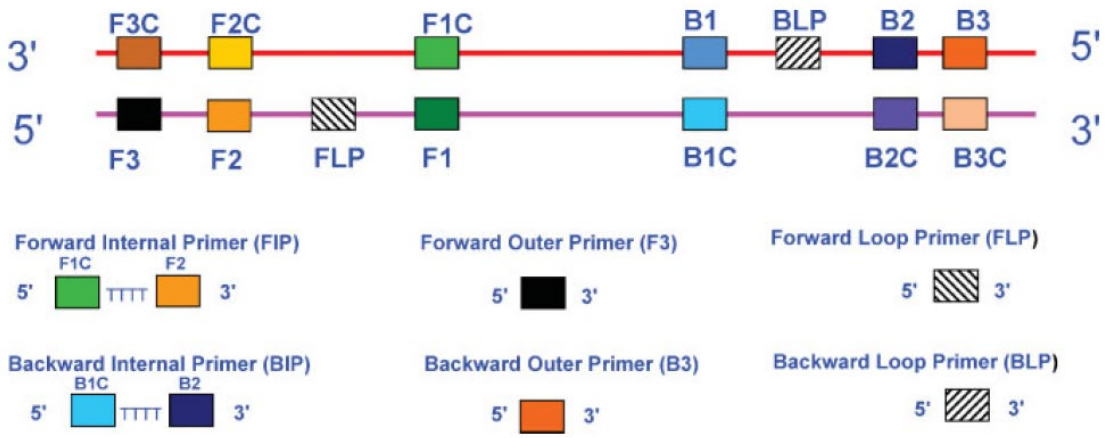

Figure 5.1: Schematic representation of a complete LAMP primer set, including loop primers and their positioning on that target DNA. Eight distinct regions are targeted, F3, F2, F1, FLP, B1, B2, B3, BLP with 'c' representing the complementary sequences of the targets. F3 and B3 are the outer primers, with FIP and BIP as the inner primers. FIP and BIP are hybrid primers, consisting of F1c and F2 sequence or B1c and B2 sequence respectively (Figure from Parida *et al*., 2008).

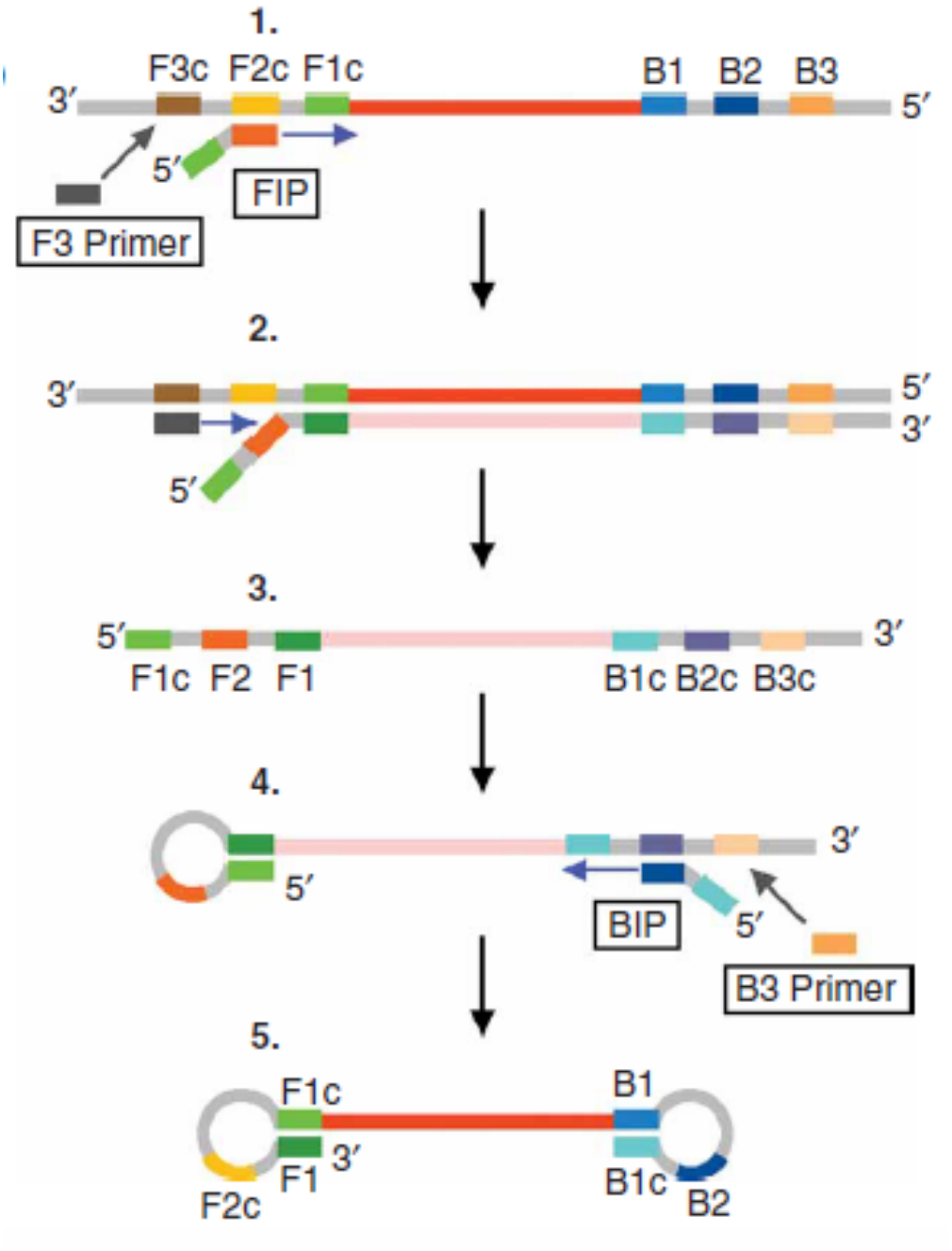

Figure 5.2: Schematic representation of non-cyclic amplification step of LAMP. Non-cyclic amplification generating the starting structure for cyclic amplification as initiated from FIP. F2 region anneals to the F2c region initiating elongation of the target DNA, see section 1. F3 Primer anneals to the F3c region on the target DNA and strand displacement DNA synthesis takes place, section 2. The DNA strand elongated from FIP is replaced and released, forming a loop structure at its 3'end, section 3. DNA synthesis continues with the ssDNA as the template with BIP and B3 in the same manner as described for FIP and F3, section 4, to generate a dumbbell-like structure with loops at each end, section 5 (Figure from Tomita *et al.*, 2008).

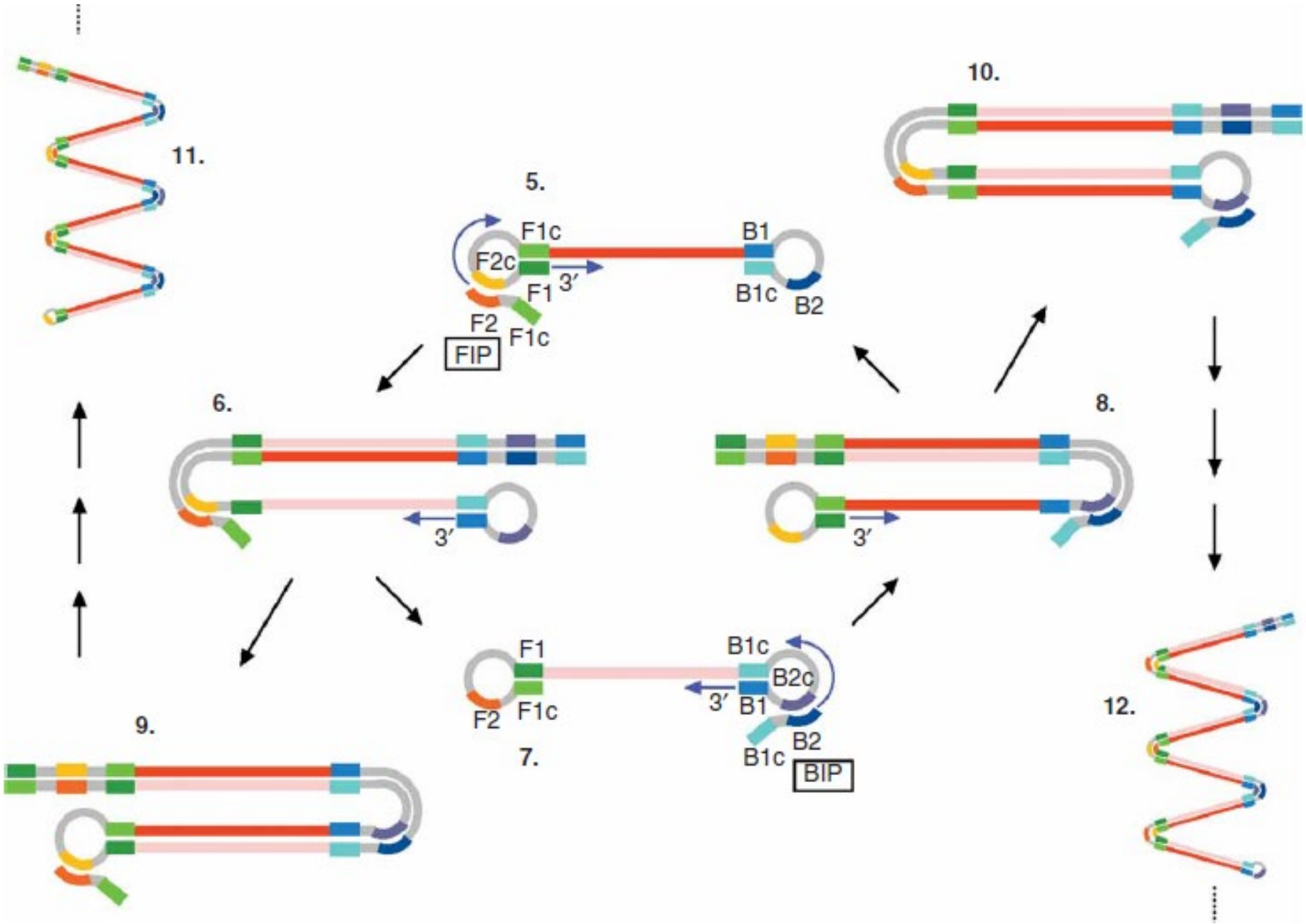
11.
5.
F1c
F2c
F1
3′
F2
F1c
FIP
B1
B1c
B2
10.
6.
3′
8.
3′
F1
F2
F1c
7.
B1c
B2c
3′
B1
B2
B1c
BIP
9.
12.

Figure 5.3, previous page: Cyclic amplification occurs with the dumbbell-like structure as the template for amplification, see Figure 5.2. Self-primed DNA synthesis starts from the 3'end of the F1 region. FIP anneals to the single strand of the F2c region in the loop structure initiating elongation, section 5. After continued amplification, section 6, the structure in section 7 is generated, complementary to the structure in section 5. Through continued amplification, section 8, the structure in section 5 is generated. This amplification cycles, generating new amplification templates, as well as more elongated structures, sections 9 - 12. The reaction is self-perpetuating, generating additional template copies, and the more elongated structures result in increased efficiency of amplification, as polymerase does not need to dissociate and bind to a new template, due to the loop templates incorporated in the elongated structure (Figure from Tomita *et al.*, 2008).

An internal primer hybridises to the loop of the dumbbell structure and initiates displacement DNA synthesis, resulting in the original stem-loop DNA and a new stem-loop that has a stem twice as long (Parida *et al*., 2008). This leads to self-primed replication resulting in continuous amplification that generates various sized structures with repeats of the target sequence within the same strand (Figure 5.3). By including loop primers within the assay (Figure 5.4), they act with a similar mechanism to the inner primers, initiating the cyclic amplification and accelerating the reaction (Figure 5.4; Nagamine

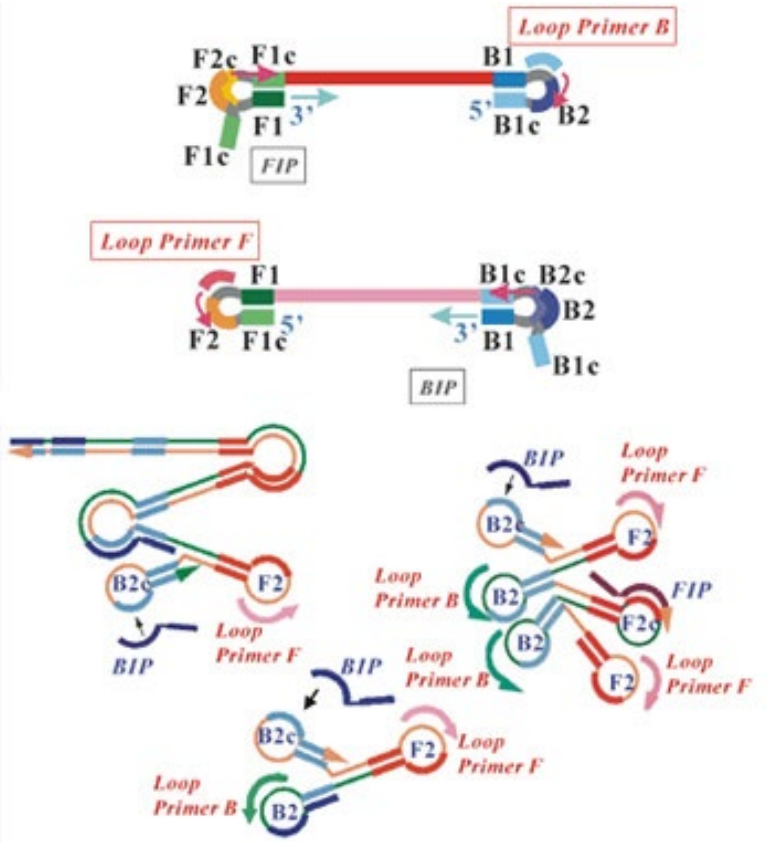

Figure 5.4: Schematic representation of loop primers generating increased amplification in LAMP. Containing sequences complementary to the single-stranded loop region within the dumbbell and elongated structures, loop primers provided an increased number of starting points for DNA synthesis in LAMP. The elongated product containing six loops, allows for six DNA synthesis start points in the presence of loop primers, whilst with the basic LAMP primer set (F3/B3 and FIP/BIP), only the two end loops could be utilised (Figure from Eiken Chemical Co. Ltd., Japan, 2005).

et al., 2002). Nagamine *et al*. (2002) found that by adding loop primers to the LAMP assay that a signal increase was seen at 14 mins, as opposed to 44 mins when the loop primers were absent.

LAMP can target and amplify target DNA with high efficiency in any genome and has been adapted to recognise DNA in bacteria (Seki *et al*., 2018; Song *et al.*, 2019), viruses (Rohatensky *et al*., 2018), parasites (Viana *et al*., 2018) and, either by combination with reverse transcription or use of OmniAmp polymerase, can be adapted to amplify RNA (Notomi *et al.,* 2000; Chander *et al*., 2014; Sabalza *et al.*, 2018). LAMP has been successfully applied to rapid and real-time detection of both DNA and RNA viruses and can be applied to a range of fields including clinical diagnosis, SNP typing, and quantification of template DNA (Parida *et al*., 2008; Rohatensky *et al*., 2018). It has been established that LAMP is effective for use in molecular point-of-care testing and the potential integration of isothermal amplification onto microchips could lead to accurate identification of disease producing genes at a patient's bed side (Parida *et al*., 2008).

Cheung and Kam (2012) noted that LAMP is more specific, rapid, simple, and less expensive to carry out than conventional PCR. Parida *et al*. (2008) noted that LAMP has the advantages of reaction simplicity and higher amplification efficiency when compared to PCR and RT-PCR. When Francois *et al*. (2011) compared the performance and robustness of LAMP assays to PCR assays, it was seen that LAMP assay yielded reproducible results through a broad range of temperatures, elongation times, and pH values, whilst PCR did not. They showed that this robustness extends to when biological samples were used without extensive DNA purification or when harsh processing reagents were used (Francois *et al*., 2011). Kaneko *et al*. (2006) noted that DNA extraction can be omitted for LAMP, whilst PCR relies on extensive DNA purification. Additionally, after sample preparation, amplification and detection of LAMP assays can be done in a single step.

Saffie *et al*. (2014) note that LAMP is a promising, reliable method for resource-limited settings. To enhance productivity, efficiency, and significantly reduce resource use in public health laboratories, rapid methods for *Salmonella* detection are needed (Cheung and Kam, 2012). Additionally, to reduce

the impact of the burden of *Salmonella*, rapid, sensitive, and reliable diagnostic tools are needed for the detection of *Salmonella* and to efficiently control the spread of infection within and between herds (Lomborg *et al*., 2007; Wattiau *et al*., 2011). This study aims to develop a loop-mediated amplification assay that is robust enough for use pen-side to cattle, with visual detection of positive results to allow for ease of use. To achieve this the following objectives were set;

- Test LAMP primer sets, generated through bioinformatic analysis, to detect multiple *Salmonella* serovars
- Develop a LAMP protocol for the visualisation of LAMP assay results with minimal to zero dependency upon apparatus
- Optimise the LAMP assay to allow for the fastest result generation, without reducing sensitivity or specificity
- Determine the robustness of the prototype LAMP assay
- Test the LAMP assay through faecal matter and optimise as required

5.2 Expanded methods for LAMP assays

Genomic DNA for the *Salmonella* strains, and *E. coli* control was purified and quantified as described in section 2.3.1. All LAMP products were visualised by gel electrophoresis as described in section 2.3.2. All LAMP assays were performed according to section 2.6, unless otherwise stated.

5.2.1 Visualisation of the LAMP assay

To enable visualisation of the LAMP assay without requiring gel electrophoresis confirmation, several visualisation methods were tested, by adapting the Optigene protocol (section 2.5.1). The dyes used within colorimetric and fluorometric assays are described in Table 5.1.

Table 5.1: The dyes used to develop a method of visualisation of the LAMP assay within this study, their mechanisms, and properties.

Dye	Dye type	Dye concentration within reactions	Colour change for positive assay in visible light	Fluorescence under UV light
Hydroxy naphthol blue	Metallochromic indicator	240µM	Violet to sky blue	No
Methylene Blue	DNA intercalating	240µM	Blue to colourless	No
Nile Blue A	DNA intercalating	240µM	Blue to colourless	No
Propidium iodide	DNA intercalating	0.06M	Dark pink to light, bright pink	Yes
SYBR Green I	DNA intercalating	400x concentration	None	Yes
SYBR Safe	DNA intercalating	400x concentration	None	Yes

5.2.1.1 Visualisation of the LAMP assay by turbidity

Methodology from Mori *et al.* (2001) was adapted to develop turbidity that could be viewed by eye, without the need for measuring apparatus. Turbidity experiments were completed as described in section 2.5.2.1, unless otherwise stated. In the final reaction volume, LAMP primers were in the

following concentrations: 0.8µM each of FIP/BIP, 0.4µM each of FLoop/Bloop, 0.2µM each of F3/B3 (Nagmine *et al*., 2002). DNA was standardised to 0.01ng/µl unless otherwise stated.

To assess the impact of additional magnesium on turbidity in the reaction mixture, a range of $MgSO_4$ concentrations from 2mM to 5.5mM in 0.5mM increments, were tested. The experiment was completed on a hotplate initially, with later amplifications being completed within a shaking incubator (100rpm) at 65°C, then terminated for 5 mins at 85°C.

Due to a lack of clear visual precipitate, template DNA was added in increasing concentrations (0.01-1ng/µl within reaction assay). Finally, after amplification was completed and the LAMP assays were terminated, assays were pulse centrifuged at maximum speed for 10 seconds to encourage precipitation of product.

5.2.1.2 Visualisation of LAMP assays using colorimetric dyes

To allow for visualisation of LAMP assay products without additional apparatus, colorimetric dyes were added. Original colorimetric dye experiments were completed as per section 2.5.2.2. Colorimetric assays were later adapted for dyes to be added after termination, see table 5.2. Assays were run for 45mins at 65°C

Table 5.2: Optigene methodology adapted from Figure 2.15, 1µl dye incorporated after reaction termination at 85°C to allow for visualisation.

Reagent	**Volume (µl)**
Turbidometric isothermal mastermix (Optigene, ISO-001t)	15
Primer mix (2x conc)	2.5
Template DNA	2
Sterile water	5.5
Total volume (µl)	25

5.2.1.3 Visualisation of LAMP assays using fluorometric dyes

To allow for visualisation of LAMP assay products, fluorometric dyes were added. Initially, fluorometric assays were completed as per section 2.5.2.3. The assays were later adapted for dyes to be added after termination, see table 5.2. Assays were run for 45mins at 65°C, then after termination at 85°C for 5 mins, the results were viewed under a UV lamp.

5.2.3 Optimisation of LAMP assay

Assays were run as described in section 5.2.1.3, unless otherwise stated. Propidium Iodide (PI) or SYBR safe (SS) were used post amplification as the visualisation dyes, unless otherwise stated.

5.2.3.1. Optimisation of LAMP assay amplification time

To determine the minimum time taken to generate a visual signal from the LAMP assay when targeting *Salmonella* sp., the assays were terminated at intervals of 15, 30, and 45mins initially, the intervals were later shortened to 15, 20, 25, 30mins. Nile Blue was used, in addition to PI and SS, as a visualisation dye for a triplicate of the time experiments at intervals 15-30mins.

5.2.3.2. Determination of optimum LAMP assay amplification temperature

To determine the temperature range in which the LAMP assay could generate a visual signal when targeting *Salmonella* sp., the LAMP assays were run at a range of temperatures starting at room temperature (~25°C) to 85°C, in increments of 10°C, for 30mins before termination and visualisation.

5.2.3.3. Specificity of LAMP assay to multiple Salmonella serovars

To determine the specificity of the primers to different *Salmonella* sp. the LAMP assay was tested against a range of *Salmonella* strains. Assays were run at an amplification temperature of 65°C for 30mins.

5.2.4 Protocol to remove LAMP amplicon contamination

Due to the prolific product generation within LAMP assays, several methods were used at once to minimise self-contamination, in addition to standard clean molecular biology practices.

- Consumables were sterilised twice
- Pipettes were cleaned thoroughly with alcohol/DNA away before and after use and left overnight under UV light
- Benches were cleaned with alcohol, air-dried for 10mins, wiped with DNA away, then bleached before and after use. Overnight the bench was left under UV light
- LAMP assays were set up within a clean hood. Hoods were turned on an hour before use and left to run, before cleaned with alcohol and left to air-dry for 10mins
- After termination, assay microtubules were placed in a microcentrifuge for a short burst to ensure the sample was at the bottom of the tube
- Assay microtubule lids were opened carefully and slowly to avoid aerosols in a location separate to the assay set-up area

5.2.5 Testing the robustness of the optimised LAMP assay

5.2.5.1. Testing LAMP assay sensitivity to low amounts of template DNA

To determine the minimum amount of DNA able to generate a visual signal from the LAMP assay, a standardised range of DNA was tested, with 6,000 copies (0.0164ng/µl) being the lowest

5.2.5.2. Testing specificity of LAMP assays on addition of scour samples

To determine the robustness of the LAMP assay, faecal matter artificially, or naturally, contaminated with *Salmonella* sp. were tested with the LAMP assay. Faecal samples were treated as per section 2.1.2, before use. Faecal samples were vortexed to ensure a uniform suspension of matter. Where applicable pipetting was used to transfer faecal samples, however in cases of solid matter a cuvette

stirrer (small plastic spatula) was used to avoid absorption of reaction mixture. Within the no template control, DNA was replaced with sterile water to complete reaction mixture volume.

To artificially contaminate *Salmonella* negative faecal samples, bacteria were prepared to the appropriate absorption as per the method stated in 2.8.1. Using a sterile microcentrifuge tube, aliquots of 90µl of scour along with 10µl of bacterial suspension were mixed to create a spiked positive sample with a known standard of bacterial number.

5.2.5.3. Testing changes in pH levels upon addition of scour samples to LAMP assays

When adding scour to assay mixtures, monitoring of pH was completed to determine whether faecal samples need to be diluted or buffered before addition to LAMP assays. Using a micro-probe, pH was monitored before scour addition, after scour addition, and after reaction termination.

5.3 LAMP results

5.3.1 LAMP assays using the Optigene protocol to detect *S.* Dublin

To establish whether the LAMP primer sets generated using bioinformatic techniques, table 2.5, could detect *Salmonella enterica* serovar Dublin (*S*. Dublin) genomic DNA, the Optigene protocol was used, as described in method section 2.5.1. All primer sets detected *S*. Dublin DNA, except bapA1.2 (Figure 5.5).

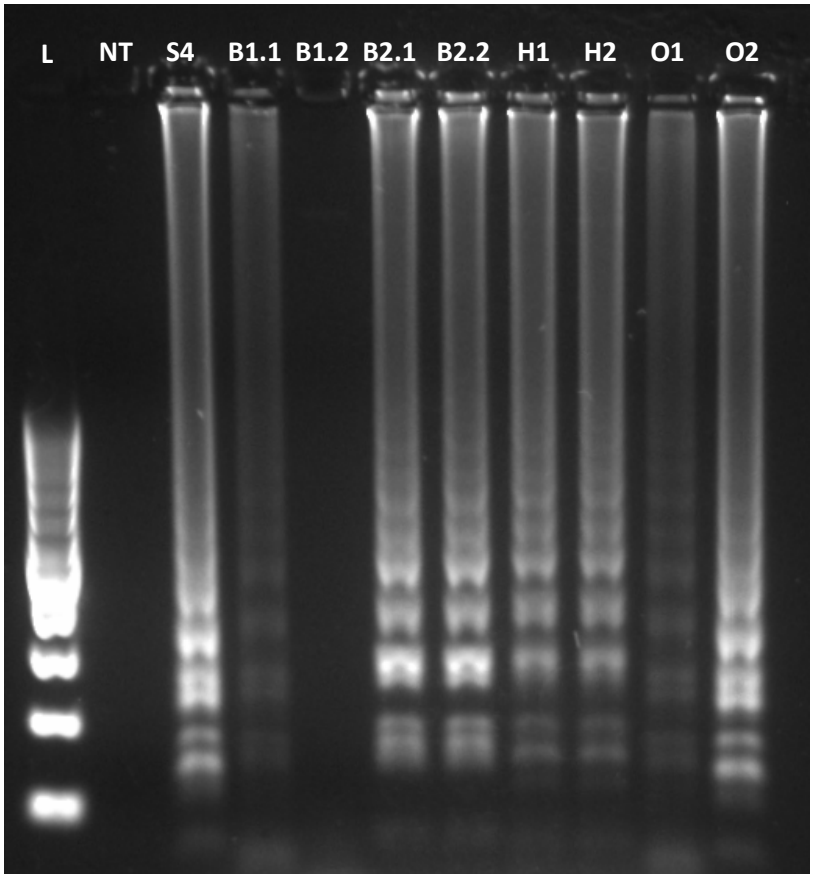

Figure 5.5: Agarose gel after electrophoresis showing LAMP assays completed using the Optigene method, against each LAMP primer set generated by bioinformatic methods, with *S*. Dublin as the target DNA.

Key: L = DNA Ladder, NT = No template control with sterile water in place of template DNA, S4 = positive control primer set, B1.1 = bapA1.1, B1.2 = bapA1.2, B2.1 = bapA2.1, B2.2 = bapA2.2, H1 = hilA1, H2 = hilA2, O1 = orgA1, O2 = orgA2

5.3.2 Visualisation of the LAMP assay by turbidity

Visualisation of the Optigene LAMP assays was initially based upon visual inspection for turbidity as described in method section 2.5.2.1, however whilst gel electrophoresis showed LAMP amplicon, no turbidity was observed. Thus, the turbidity LAMP assays were optimised as described in section 5.2.1.1. Increased concentrations of template DNA were used (1ng/µL *versus* 0.1ng/µL), producing observable amounts of product when observed via gel electrophoresis (Figure 5.6A), however little to no visual turbidity was observed within reaction tubes after centrifugation (Figure 5.6B).

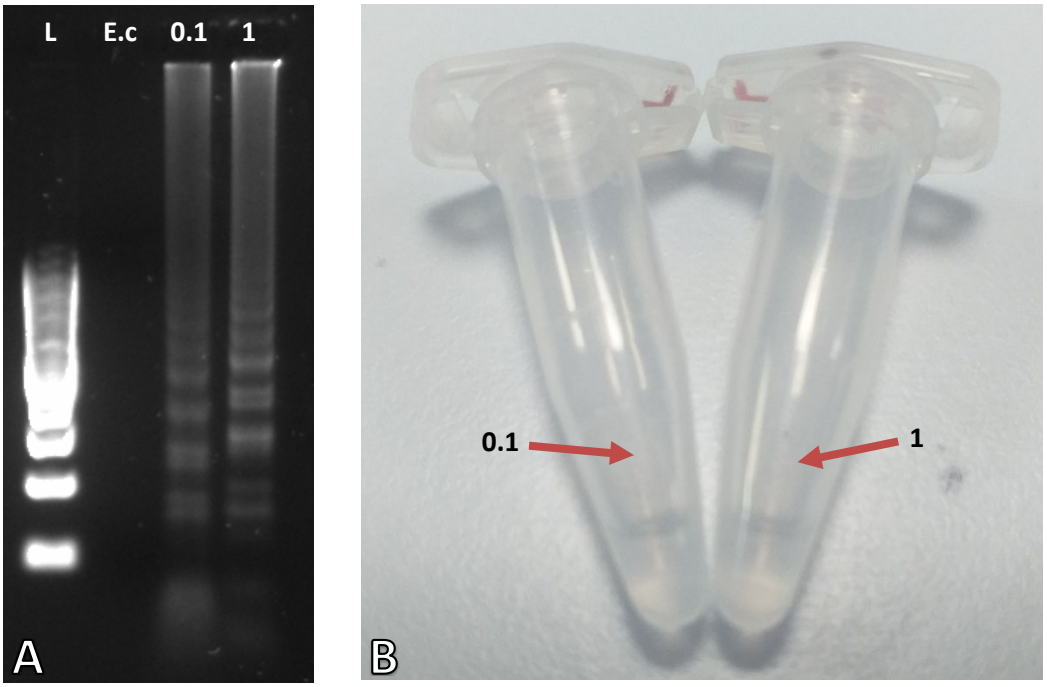

Figure 5.6: (A) Agarose gel showing turbidity LAMP assays using the orgA1 primer set with an increase in *S*. Dublin template DNA. (B) turbidity LAMP assay reaction tubes after burst centrifuging targeting increased concentrations of *S.* Dublin DNA.

Key: L = DNA Ladder, E.c = Negative control assay using *E. coli* DNA as a template, 0.1 = 0.1ng/µl of template DNA within the reaction tube (100,000 DNA copies), 1 = 1ng/µl of template DNA within the reaction tube (1,000,000 DNA copies)

5.3.3 Visualisation of LAMP assays using colorimetric dyes

Colorimetric assays were initially completed as described in methods section 2.5.2.2. No colour change was observed after amplification with methylene blue or hydroxy naphthol blue as a colorimetric dye, thus they were no longer tested. No colour change, nor amplification product, was observed with any intercalating dyes after amplification and it was established that dyes that intercalated DNA needed to be added after amplification and termination of LAMP, due to interference with the function of the strand displacing DNA polymerase, thus the protocol was optimised as described in section 5.2.1.2.

Once added after termination of LAMP assays, Nile Blue showed colour change within the Sal4 positive control, a darker blue than the no template and *E. coli* negative controls, when amplified for ≤45 minutes (Figure 5.7), no change was seen with the LAMP primer sets generated within this study. For the LAMP primer sets generated in this study no discernible colour change was observed within reaction tube, however amplification of LAMP products can be seen via gel electrophoresis for assays tested with Nile Blue (Figure 5.8),

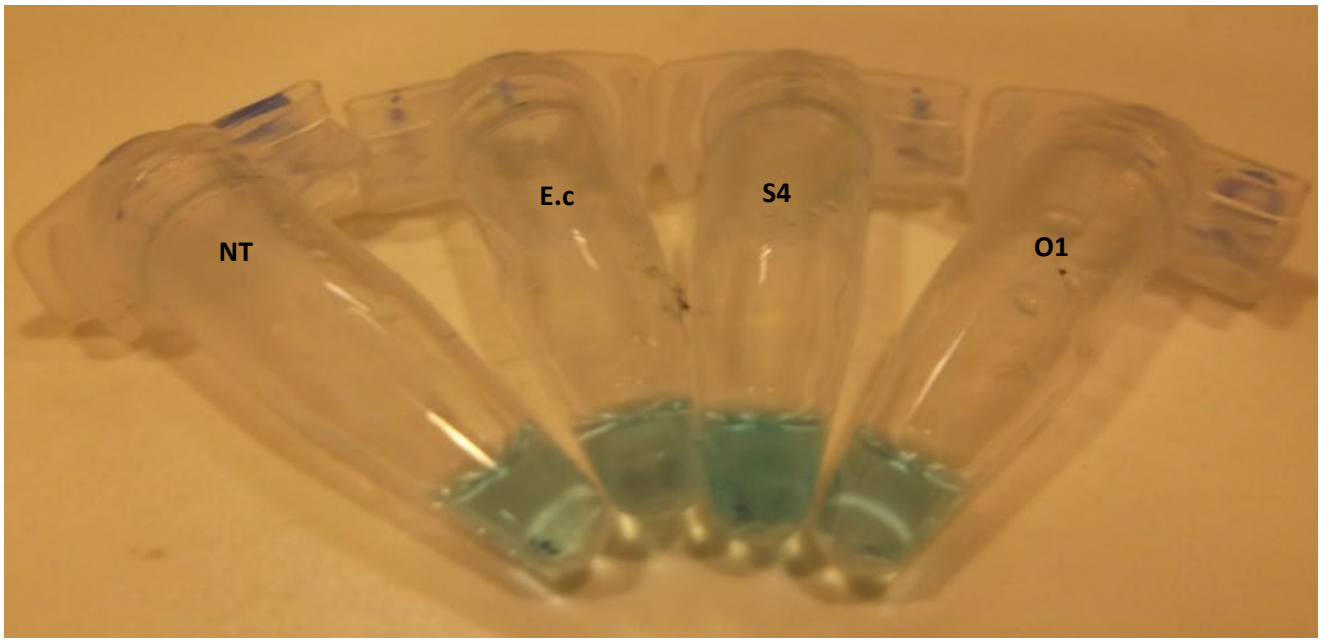

Figure 5.7: Colorimetric LAMP assay reaction tubes after 45mins amplification, targeting *S*. Dublin, and Nile blue added after termination. Obvious visual colour change was only observed at 45mins within the Sal4 positive control primer set, despite product being seen from the positive control and test sample on the subsequent gel.

Key: NT = No template control with sterile water in place of template DNA, E.c = Negative control assay using *E. coli* DNA

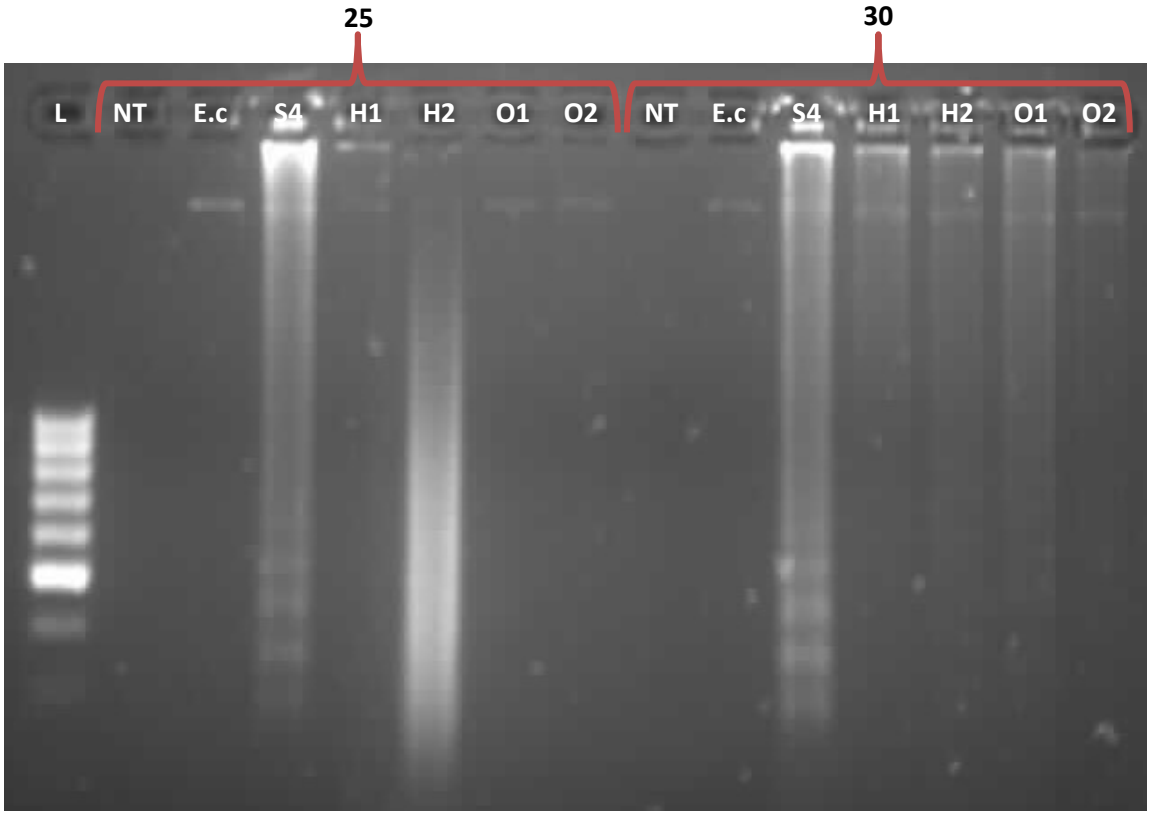

Figure 5.8: Agarose gel showing colorimetric LAMP assays terminated after 25 and 30mins amplification. Whilst Nile Blue was added after termination, no visible colour change was seen in reaction tubes, however product is visible for the positive control and H2 primer sets at 25mins and all primer sets at 30mins.

Key: L = DNA Ladder, NT = No template control with sterile water in place of template DNA, E.c = Negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, H1 = hilA1, H2 = hilA2, O1 = orgA1, O2 = orgA2, 25 = 25 minutes amplification time, 35 = 35 mins amplification time

With propidium iodide, a visual change, pink to a brighter pink, could be seen between negative and positive controls at 45mins (Figure 5.9). No colour change was seen at less than 45mins despite visible amplification being apparent on the agarose gel. With SYBR safe, no visual colour change was seen at any time point, despite amplification being apparent on the agarose gel after electrophoresis (Figure 5.10).

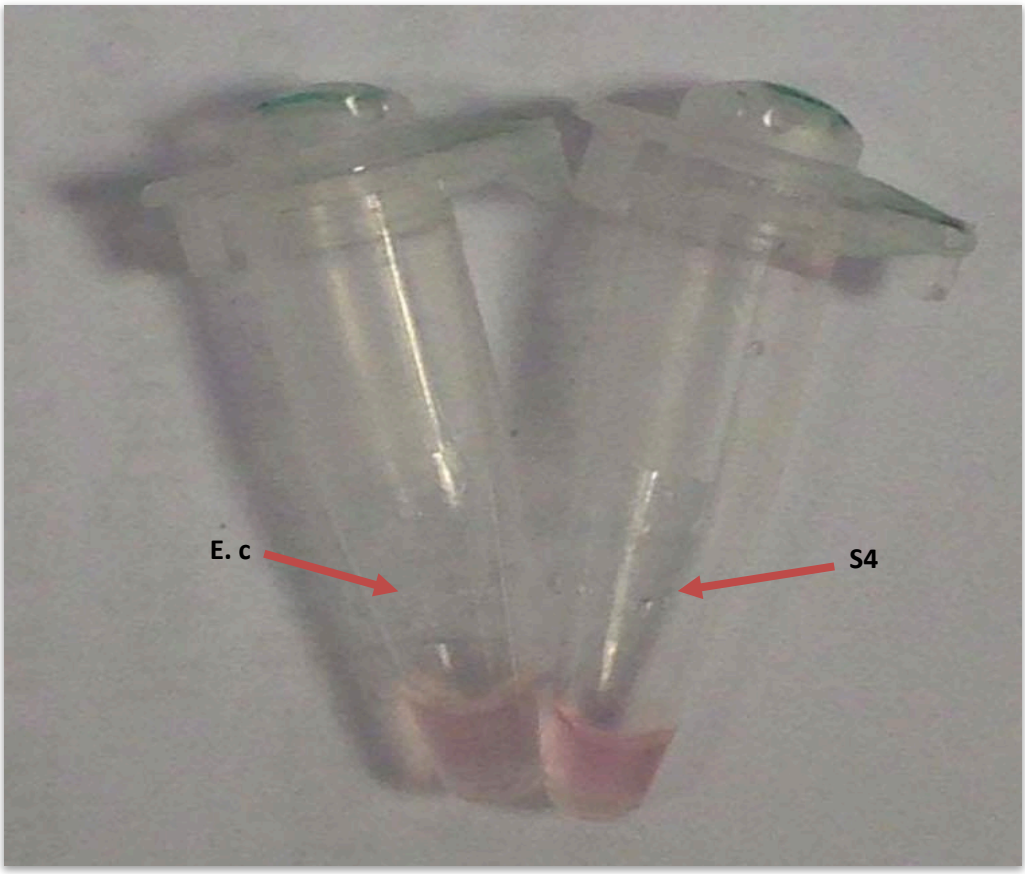

Figure 5.9: Colorimetric LAMP assay reaction tubes 45mins amplification, with propidium iodide added after termination.

Key: E.c = Negative control assay using *E. coli* DNA as a template, S4 = positive control primer set,

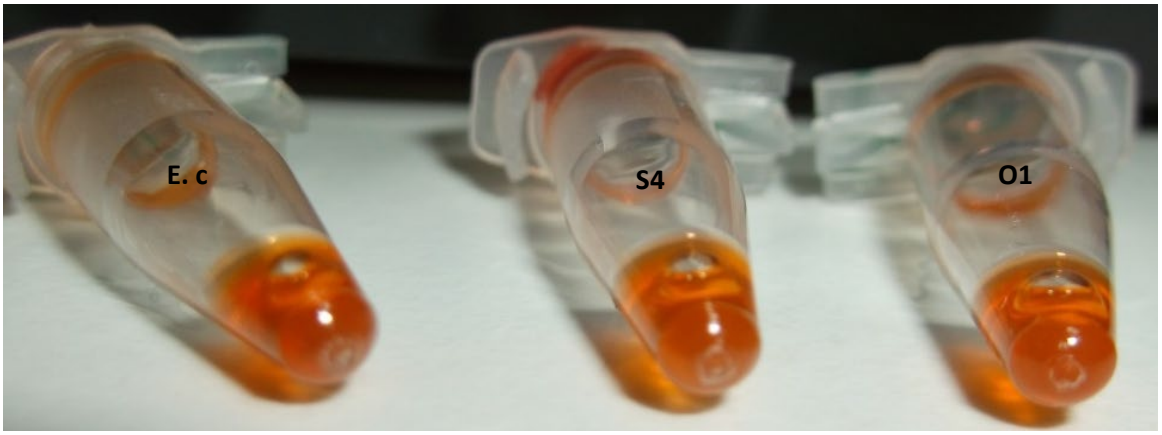

Figure 5.10: Colorimetric LAMP assay reaction tubes 45mins amplification, with SYBR safe added after termination.

Key: E.c = Negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, O1 = orgA1 primer set

5.3.4 Visualisation of LAMP assays using fluorometric dyes

Fluorometric LAMP assays were initially completed as described in 2.5.2.3, which yielded no fluorescence. No amplification products were observed on agarose gels after electrophoresis. Thus, the fluorometric LAMP assays were optimised as described in 5.2.1.3, with dyes being added after termination of the reaction. Using this protocol, amplification was observed in gels and fluorescence was seen under UV light.

For each assay run, a positive fluorescence response was considered a visible change in light/colour emission under UV light. A negative fluorescence response was a lack of colour/light change. A positive fluorescence response was assigned a nominal value of 1 and no response was assigned 0, to allow for numerical determination of overall fluorometric response.

In LAMP assays with amplicon present, Propidium iodide showed clear bright pink fluorescence when added (Figure 5.11), compared to the dull pink colour observed without amplicon present. SYBR safe produced a yellow/light orange fluorescence (figure 5.12), when amplicon was present and remained red when amplicon was not present. With SYBR Green I, bright green fluorescence (Figure 5.13) was seen in the presence of amplicon and remained clear of colour when amplicon was not present.

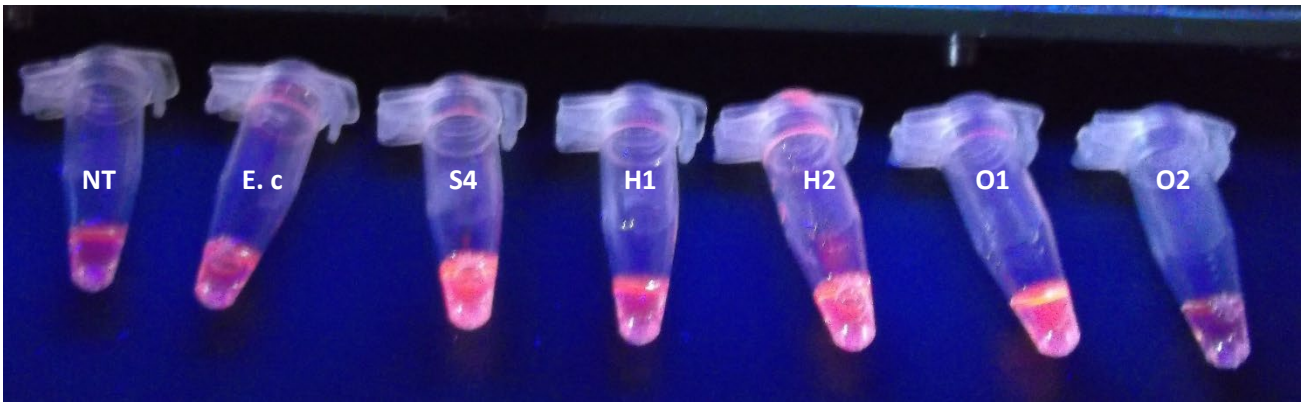

Figure 5.11: Fluorometric LAMP assay reaction tubes under UV light, after 25 minutes amplification with propidium iodide added after termination, showing clear positive signals for S4, H1, H2, and O1.

Key: NT = No template control with sterile water in place of template DNA, E.c = Negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, H1 = hilA1 primer set, H2 = hilA2 primer set, O1 = orgA1 primer set, O2 = orgA2 primer set,

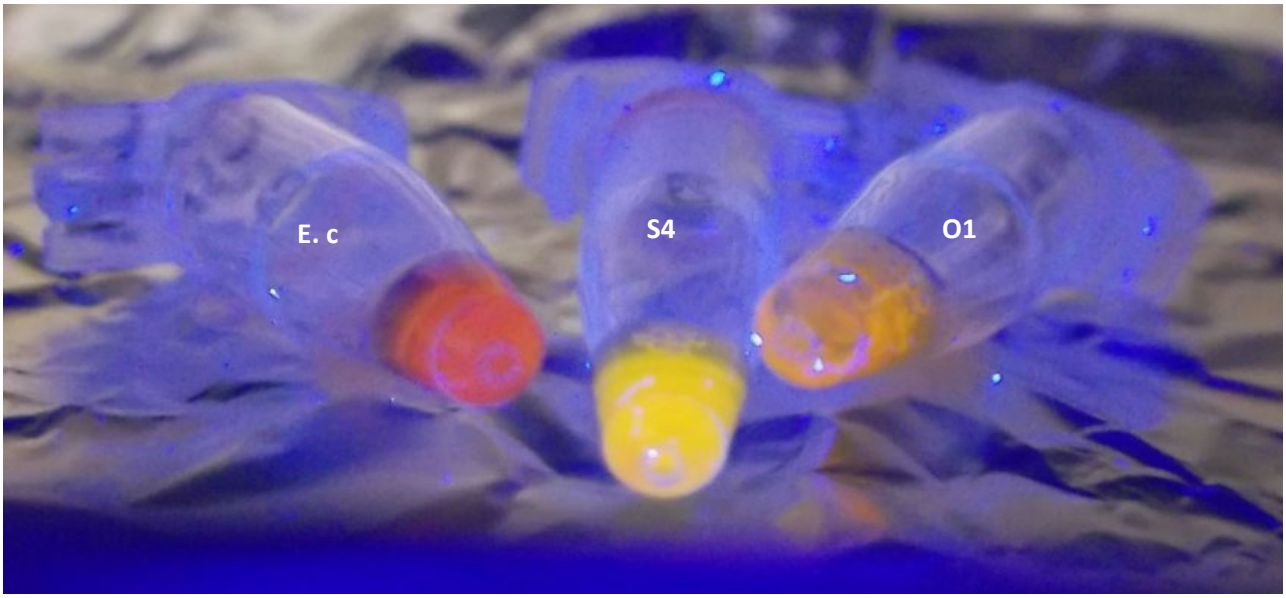

Figure 5.12: Fluorometric LAMP assay reaction tubes under UV light, after 45 minutes amplification with SYBR safe added after termination. Compared to the negative *E. coli* control, lightening of colour due to fluorescence seen for S4 and O1.

Key: E.c = Negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, O1 = orgA1 primer set

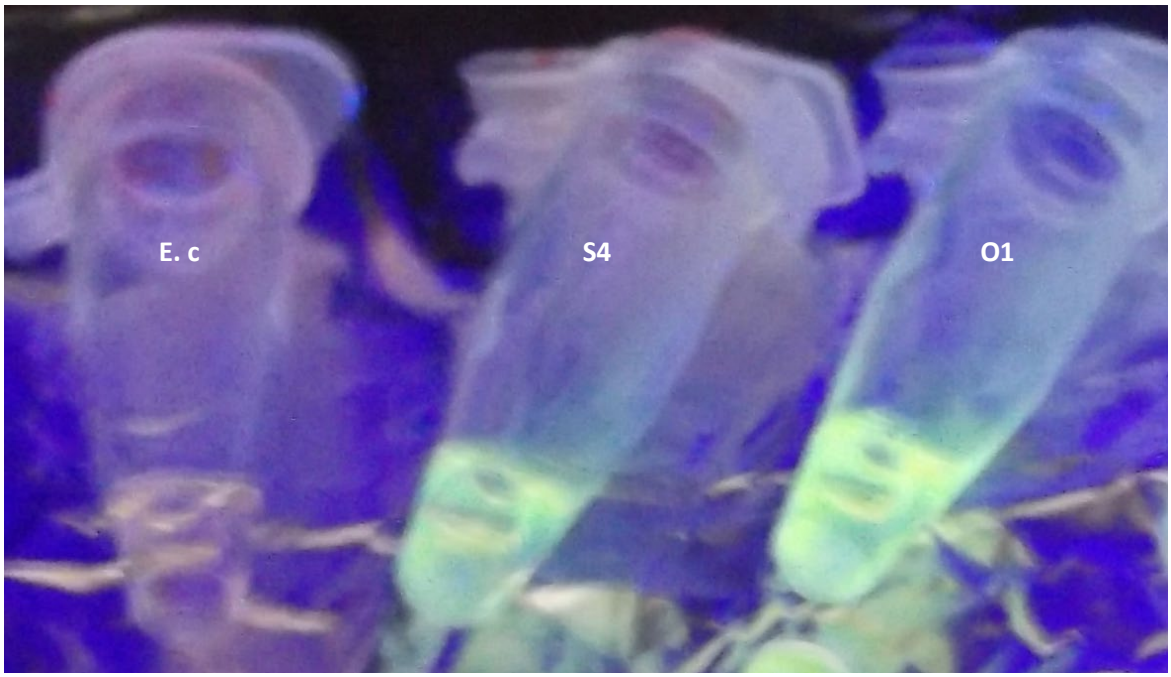

Figure 5.13: Fluorometric LAMP assay reaction tubes under UV light, after 45 minutes amplification with SYBR Green 1 added after termination. Clear green fluorescence can be seen for S4 and O1.

Key: E.c = Negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, O1 = orgA1 primer set

Agarose gels showed product that corresponded with the fluorescence observed, addition of dyes did not interfere with gel electrophoresis (Figure 5.14).

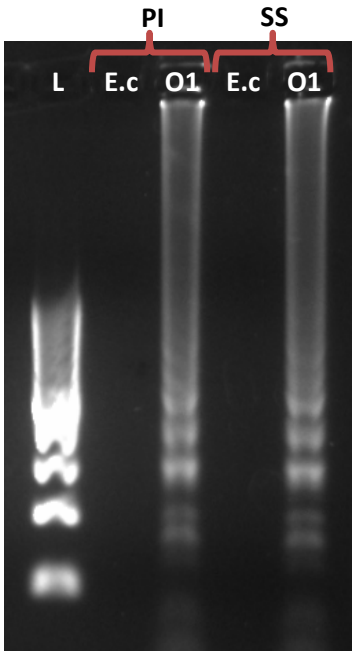

Figure 5.14: orgA1 LAMP assay reaction products on an agarose gel after 45 mins amplification and fluorometric dyes added after termination

Key: L = DNA Ladder, E.c = negative control assay using *E. coli* DNA as a template, O1 = orgA1 primer set, PI = propidium iodide, SS = SYBR safe

Examining Figures 5.15, 5.16, and 5.17, from the gel (Figure 5.15) and corresponding fluorometric LAMP reaction tubes using propidium iodide (Figure 5.16), propidium iodide fluoresced when amplicon was visible on the gel at all time points. For PI added after 30 minutes amplification (Figure 5.15 & 5.16), LAMP amplicon ladder bands were seen for the no template DNA control (NT), the negative control containing *E. coli* genomic DNA (E. c), Sal4 primer set (S4), hilA1 primer set (H1), and orgA1 (O1) primer set. Fluorescence can be seen in the corresponding reaction tubes, although the response in NT and E.c is observably lower than the Sal4 positive control. For SYBR safe, where amplicon was visible on the gel (Figure 5.15), fluorescence (Figure 5.17) was not always seen when amplicon ladder bands were faint. For example, when SS was added after 20 minutes amplification (Figures 5.15 & 5.17), LAMP amplicon ladder patterns were seen for S4 and faintly for H1 and H2, however fluorescence was only observed in the S4 lamp reaction tube (Figure 5.17).

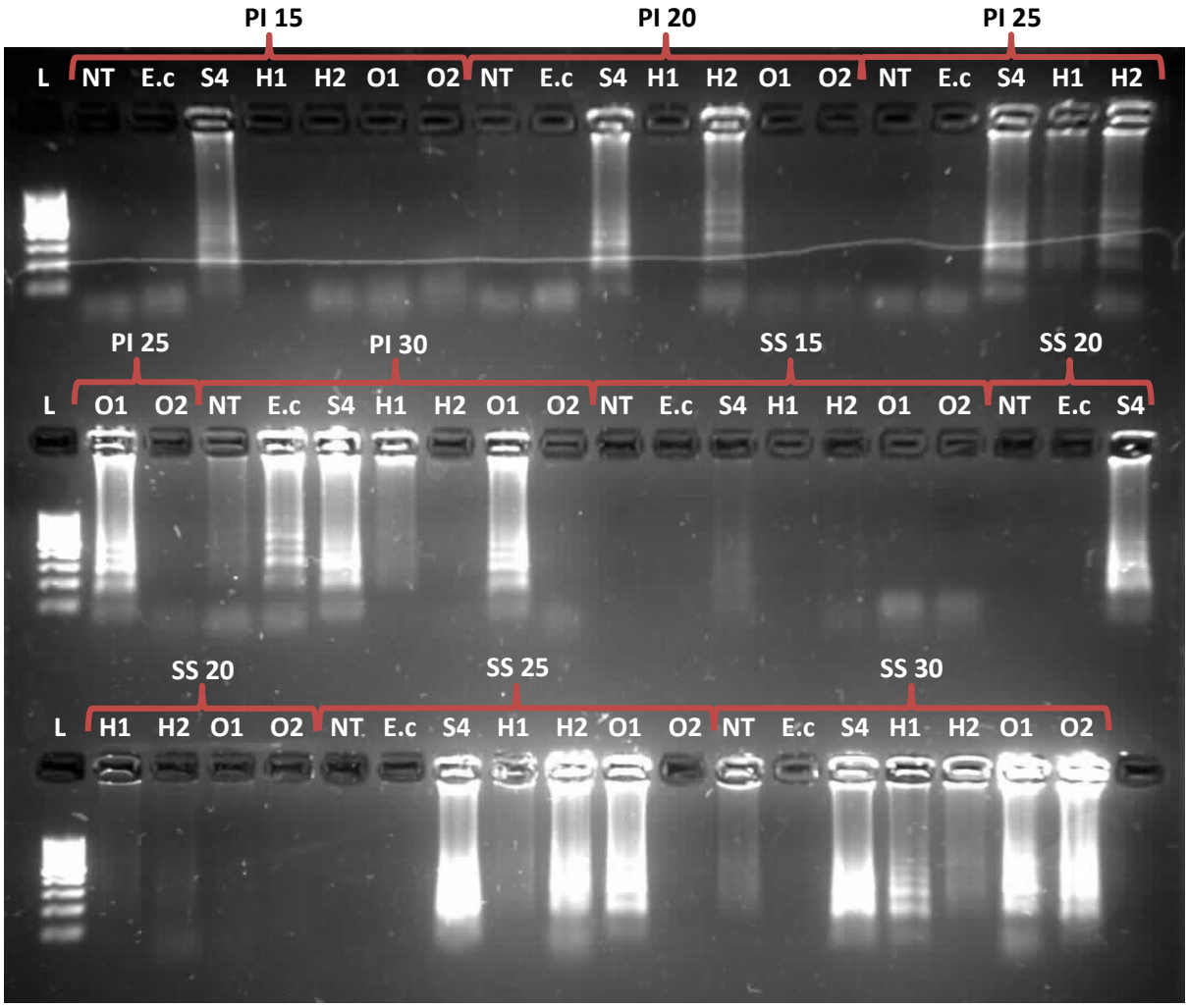

Figure 5.15: Agarose gel showing the results of two time optimisation LAMP assays, with fluorometric detection, using either propidium iodide or SYBR safe, after termination.

Key: L = DNA Ladder, NT = No template control with sterile water in place of template DNA, E.c = negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, H1 = hilA1 primer set, H2 = hilA2 primer set, O1 = orgA1 primer set, O2 = orgA2 primer set, PI = propidium iodide, SS = SYBR safe, 15 = 15 minutes amplification time, 20 = 20 minutes amplification time, 25 = 25 minutes amplification time, 30 = 30 minutes amplification time

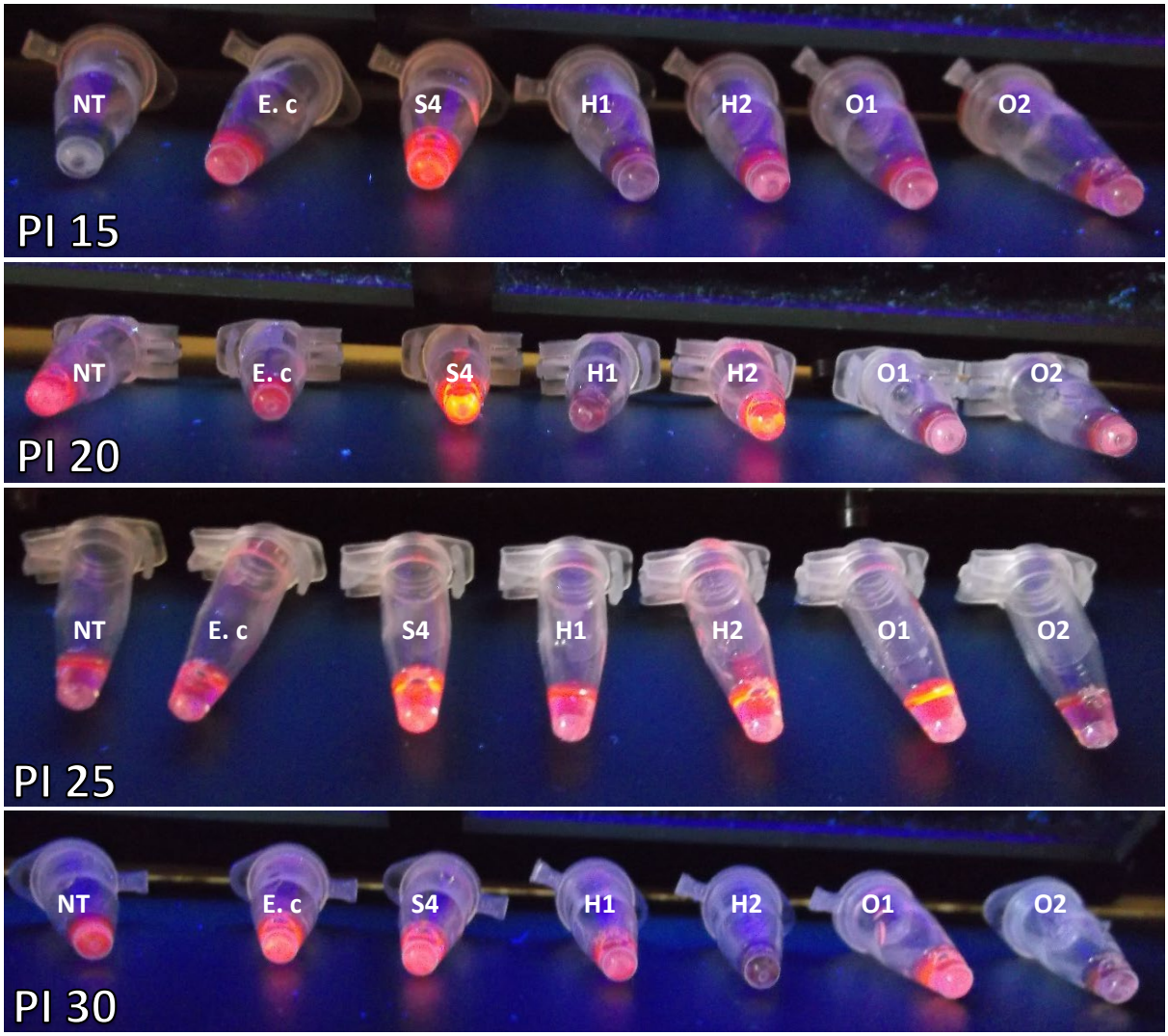

Figure 5:16: Time optimisation LAMP reaction tubes corresponding with electrophoresis results seen in 5.14. Propidium iodide used to visualise amplification under UV light.

Key: NT = No template control assay with sterile water in place of template DNA, E.c = negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, H1 = hilA1 primer set, H2 = hilA2 primer set, O1 = orgA1 primer set, O2 = orgA2 primer set, PI = propidium iodide, 15 = 15 minutes amplification time, 20 = 20 minutes amplification time, 25 = 25 minutes amplification time, 30 = 30 minutes amplification time

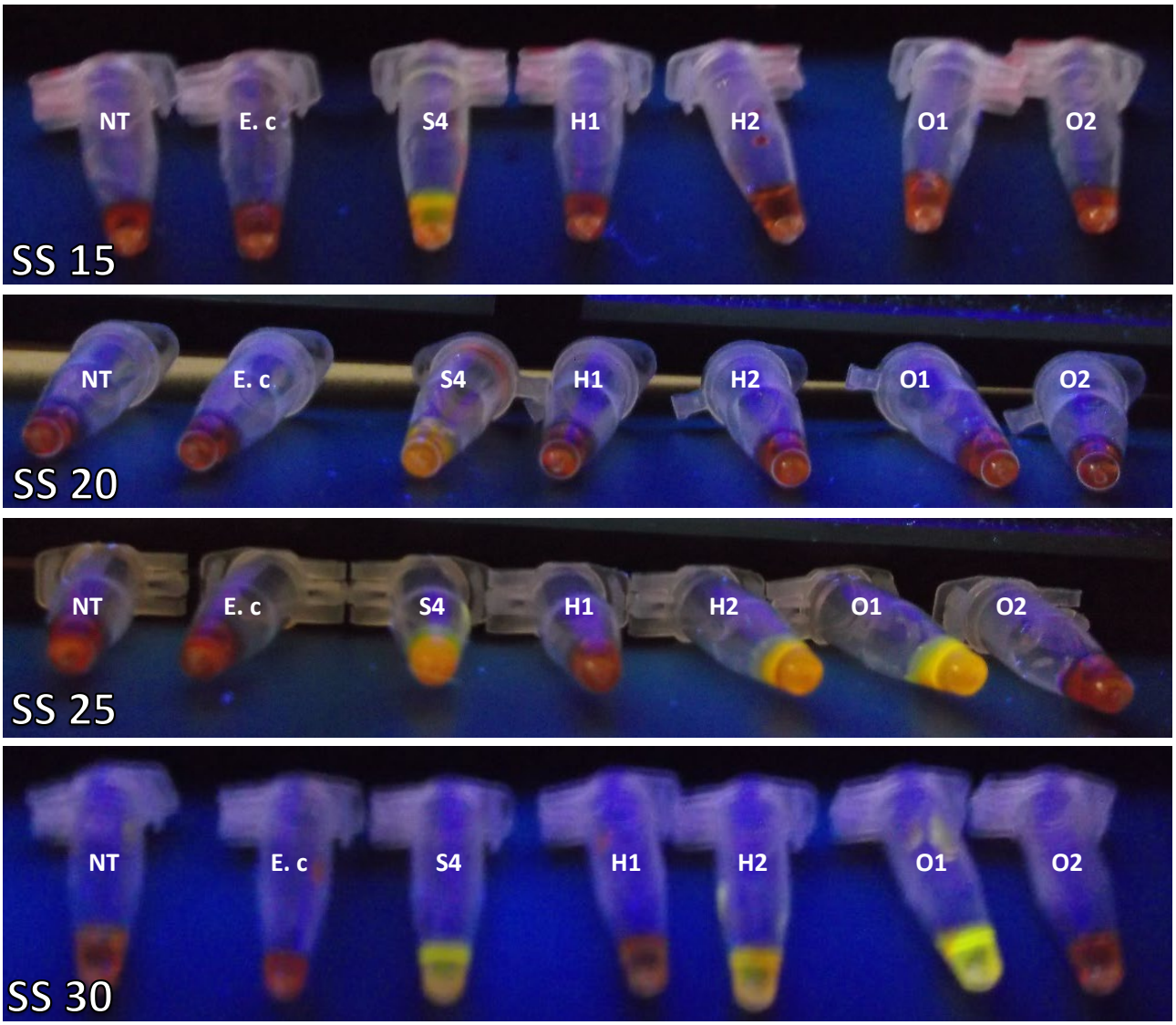

Figure 5:17: Time optimisation LAMP reaction tubes corresponding with electrophoresis results seen in 5.14. SYBR safe used to visualise amplification under UV light.

Key: NT = No template control with sterile water in place of template DNA, E.c = negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, H1 = hilA1 primer set, H2 = hilA2 primer set, O1 = orgA1 primer set, O2 = orgA2 primer set, SS = SYBR safe, 15 = 15 minutes amplification time, 20 = 20 minutes amplification time, 25 = 25 minutes amplification time, 30 = 30 minutes amplification time

5.3.5 Optimisation of amplification time of the fluorometric LAMP assay

Amplification time was tested as described in section 5.2.3.1. Positive control primer set, Sal 4, and the bapA2.1 (B2.1) primer set consistently showed strong amplification from as early as 15mins (Table 5.3 & Table 5.4). Faint amplicon products could be seen for hilA2 (H2), orgA1 (O1) and orgA2 (O2) at

15 mins but was not consistent (Figure 5.15). At 30 minutes amplification was seen for all primer sets, with B2.1, *hilA* and *orgA* primer sets having strong amplicon products (Table 5.3 &Table 5.4). BapA1.1 (B1.1), bapA1.2 (B1.2) and bapA2.2 (B2.2) had weak amplicon bands at 30mins (Figure 5.15).

When measuring the LAMP assays with fluorescence at different time points, B2.1 showed the strongest response compared to other test primer sets, producing a strong response at 20mins with both PI and SS (Table 5.3 & 5.4). B1.1, B1.2, and B2.2 produced a weak response with PI at 25mins, however showed no response at 30mins (Table 5.3). With SS, B1.1, B1.2, and B2.2 produced a weak response at 30mins, with no response at time points below (Table 5.4). In general, longer amplification times generated a great fluorescent response, however when using PI as the endpoint dye, 25 minutes amplification produced a greater fluorescent response.

Table 5.3: The averaged results of visible fluorescence from Optigene LAMP assays with varying amplification times. Fluorometric indicator used was propidium iodide, added after assay termination (n=3). Standard deviation values are displayed in brackets.

Time (mins)	**Primer set**										
	NT	Negative	Positive	B1.1	B1.2	B2.1	B2.2	H1	H2	O1	O2
15	0	0	0.83 (0.26)	0	0	0.83 (0.35)	0	0	0.5 (0)	0.38 (0.25)	0.63 (0.25)
20	0	0	0.8 (0.45)	0	0	1 (0)	0	0	0.67 (0.29)	0.67 (0.29)	0.67 (0.29)
25	0	0	1 (0)	0.17 (0.29)	0.17 (0.29)	1 (0)	0.33 (0.58)	0.38 (0.48)	0.88 (0.25)	0.88 (0.25)	0.38 (0.48)
30	0	0	1 (0)	0	0	1 (0)	0	0.33 (0.58)	0.83 (0.29)	0.83 (0.29)	0.83 (0.29)

Legend:

	Strong response (fluorescence = 1)
	Medium response (fluorescence = ≥0.5)
	Weak response (fluorescence = <0.5)
	No response (fluorescence = 0)

Table 5.4: The averaged results of visible fluorescence from Optigene LAMP assays with varying amplification times. Fluorometric indicator used was SYBR safe, added after assay termination (n=3). Standard deviation values are displayed in brackets.

Time (mins)	Primer set										
	NT	Negative	Positive	B1.1	B1.2	B2.1	B2.2	H1	H2	O1	O2
15	0	0	1 (0)	0	0	0.33 (0.58)	0	0	0	0	0
20	0	0	1 (0)	0	0	1 (0)	0	0	0.25 (0.5)	0.63 (0.48)	0.13 (0.25)
25	0	0	1 (0)	0	0	1 (0)	0	0.13 (0.25)	1 (0)	1 (0)	0.5 (0.58)
30	0	0	1 (0)	0.67 (0.58)	0.17 (0.29)	1 (0)	0.5 (0.5)	0	0.83 (0.29)	0.83 (0.29)	0.5 (0.5)

Legend:	
	Strong response (fluorescence = 1)
	Medium response (fluorescence = ≥0.5)
	Weak response (fluorescence = <0.5)
	No response (fluorescence = 0)

5.3.6 Optimisation of amplification temperature of the fluorometric LAMP assay

Amplification temperature optimisation of LAMP assays was completed as described in section 5.2.3.2. No amplification was observed for any primer set on agarose gels when the amplification temperature was ≤35°C. After electrophoresis, weak ladder patterns were seen for B2.1, H2 and *orgA* primer sets, after amplification at 45°C (Figure 5.18). On agarose gels, after amplification at 55°C and 65°C, ladder bands was seen for B2.1, H2 and *orgA* primer sets with 65°C being optimal (Figure 5.19 and 5.20). At 75°C LAMP ladder patterns were weak (Figure 5.21) and at 85°C no amplification was observed after electrophoresis.

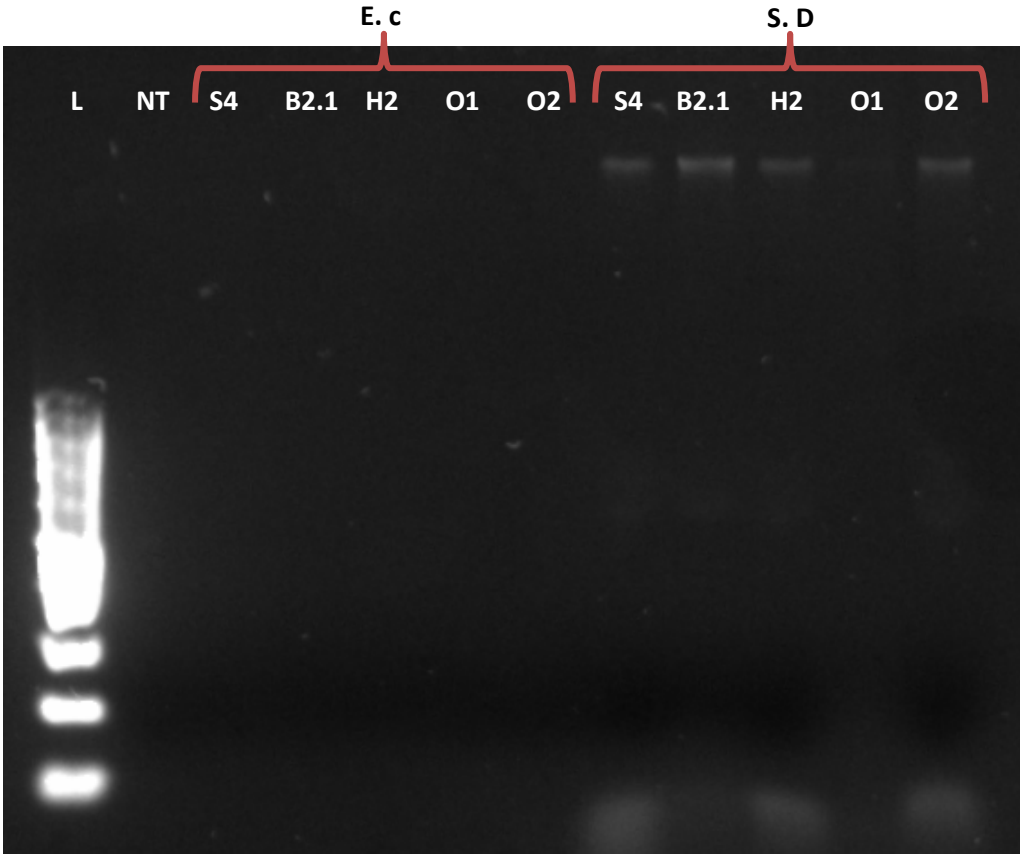

Figure 5:18: Fluorometric LAMP assay product on an agarose gel, after 30 minutes amplification at 45°C

Key: L = DNA Ladder, NT = No template control assay with sterile water in place of template DNA, E.c = negative control assay using *E. coli* DNA as a template, S.D = *S.* Dublin used as the DNA template, S4 = positive control primer set, B2.1 = bapA2.1 primer set, H2 = hilA2 primer set, O1 = orgA1 primer set, O2 = orgA2 primer set.

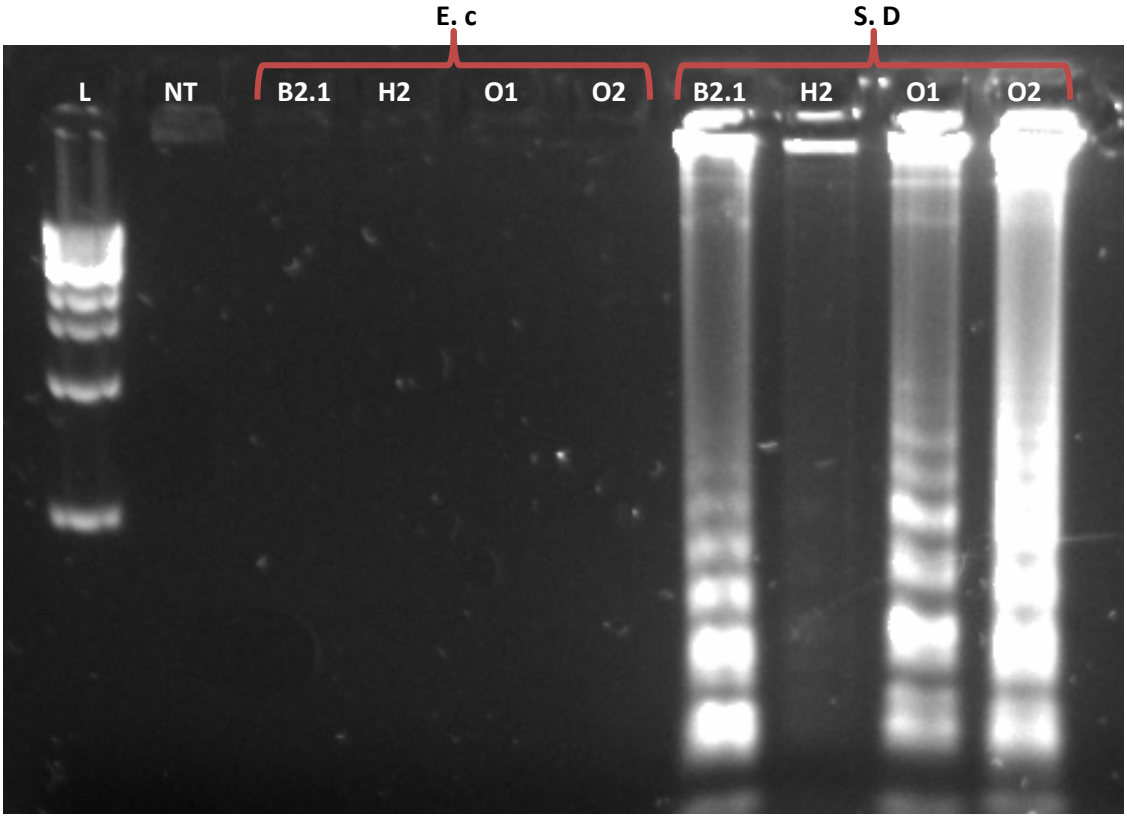

Figure 5:19: Fluorometric LAMP assay product on an agarose gel, after 30 minutes amplification at 55°C

Key: L = DNA Ladder, NT = No template control with sterile water in place of template DNA, E.c = negative control assay using *E. coli* DNA as a template, S.D = *S.* Dublin used as the DNA template, S4 = positive control primer set, B2.1 = bapA2.1 primer set, H2 = hilA2 primer set, O1 = orgA1 primer set, O2 = orgA2 primer set

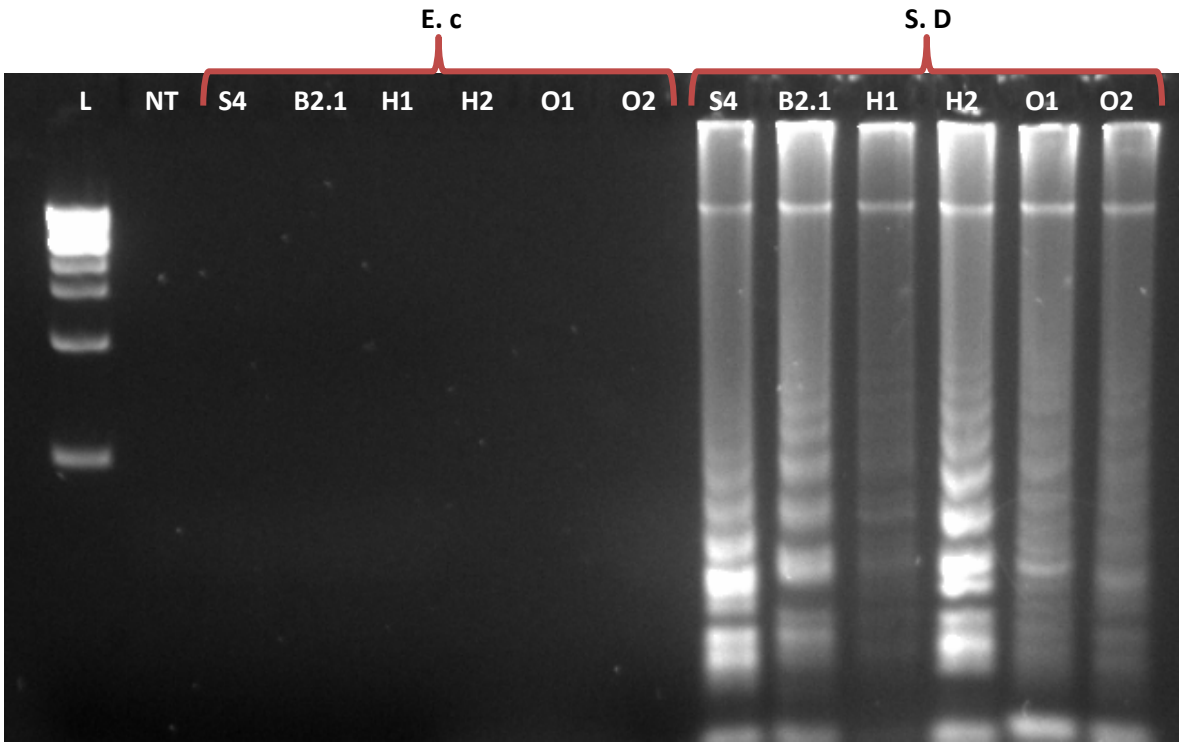

Figure 5:20: Fluorometric LAMP assay product on an agarose gel, after 30 minutes amplification at 65°C

Key: L = DNA Ladder, NT = No template control with sterile water in place of template DNA, E.c = negative control assay using *E. coli* DNA as a template, S.D = *S.* Dublin used as the DNA template, S4 = positive control primer set, B2.1 = bapA2.1 primer set, H1 = hilA1 primer set, H2 = hilA2 primer set, O1 = orgA1

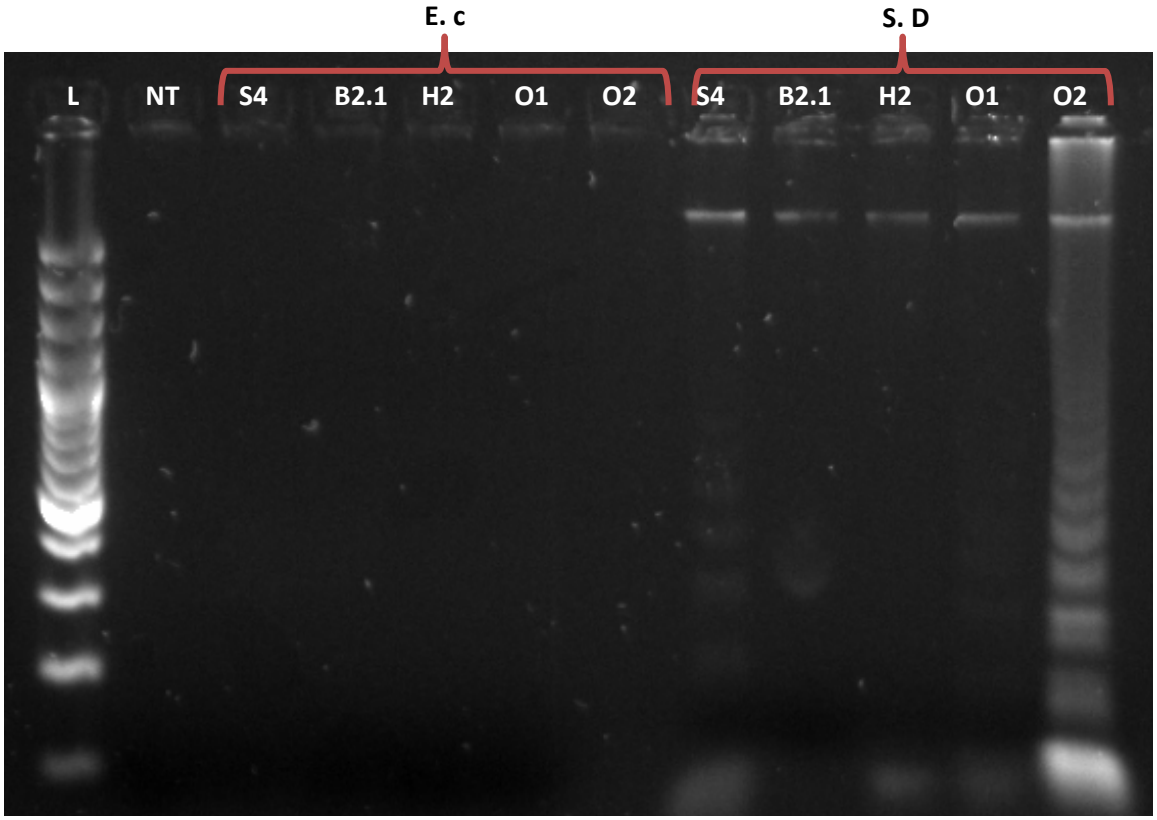

Figure 5:21: Fluorometric LAMP assay product on an agarose gel, after 30 minutes amplification at 75°C

Key: L = DNA Ladder, NT = No template control with sterile water in place of template DNA, E.c = negative control assay using *E. coli* DNA as a template, S.D = *S.* Dublin used as the DNA template, S4 = positive control primer set, B2.1 = bapA2.1 primer set, H2 = hilA2 primer set, O1 = orgA1 primer set, O2 = orgA2 primer set

When visualised with propidium iodide, strongest fluorescence results were seen at 55-65°C overall. The strongest fluorescence for B2.1 was seen at 65°C, with a medium response at 55°C and weak responses at 45°C and 75°C (Table 5.5). O2 showed a weak fluorescence response from 45-75°C, with no clear optimum temperature (Table 5.5). A fluorescence response was seen at only 55-65°C for H2 (Table 5.5). O1 produced a weak fluorescence response at 45°C, and a medium response at 55-65°C.

Table 5.5: The averaged results of visible fluorescence from optimised LAMP assays performed at different temperatures (n=5). Standard deviation values are displayed in brackets.

Temp (°C)	Primer Set						
	NT	Negative	Positive	B2.1	H2	O1	O2
25	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0
45	0	0	0.2 (0.45)	0.125 (0.25)	0	0.1 (0.22)	0.1 (0.22)
55	0	0	0.6 (0.55)	0.625 (0.48)	0.7 (0.45)	0.5 (0.5)	0.2 (0.45)
65	0	0	0.6 (0.55)	1 (0)	0.5 (0.5)	0.5 (0.5)	0.2 (0.45)
75	0	0	0.2 (0.45)	0.125 (0.25)	0	0	0.2 (0.45)
85	0	0	0	0	0	0	0

Legend:	
	Strong response (fluorescence = 1)
	Medium response (fluorescence = ≥0.5)
	Weak response (fluorescence = <0.5)
	No response (fluorescence = 0)

5.3.7 The specificity of the generated LAMP primer sets against different *Salmonella* serovars

Assays were completed as described in section 5.2.3.3, against a panel of *Salmonella* genomic DNA. *Salmonella* serovars included; *Salmonella enterica* serovar Agama (*S.* Agama), *S.* Dublin, *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis), *Salmonella enterica* serovar Mbandaka (*S.* Mbandaka), *Salmonella enterica* serovar Montevideo (*S.* Montevideo), *Salmonella enterica* serovar Newport (*S.* Newport, and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). Specificity results were gained for B2.1 and *orgA* primer sets in duplicate. Due to LAMP amplicon contamination, triplicates were not completed, and *hilA* primer sets were not tested, due to contamination issues (Section 5.3.8).

LAMP primer sets bapA2.1 and orgA1 recognised all *Salmonella* serovars tested (Figure 5.22 & Figure 5.23). OrgA2 recognised all serovars accept *S*. Mbandaka (Figure 5.24).

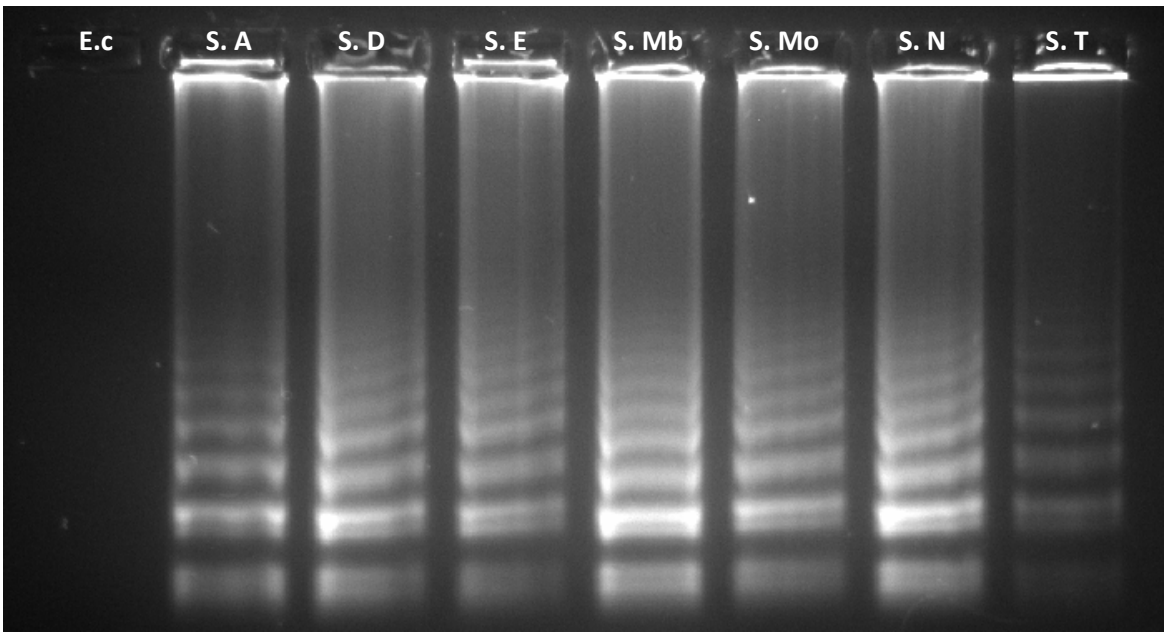

Figure 5.22: Agarose gel showing products of bapA2.1 LAMP assay amplified for 30 minutes at 65°C targeting multiple *Salmonella* serovars. NT assays showed no product.

Key: E.c = negative control assay using *E. coli* DNA as a template, S. A = *S*. Agama genomic DNA, S. D = *S.* Dublin genomic DNA, S. E = *S.* Enteritidis, S. Mb = *S*. Mbandaka genomic DNA, S. Mo = *S.* Montevideo genomic DNA, S. T = *S.* Typhimurium genomic DNA

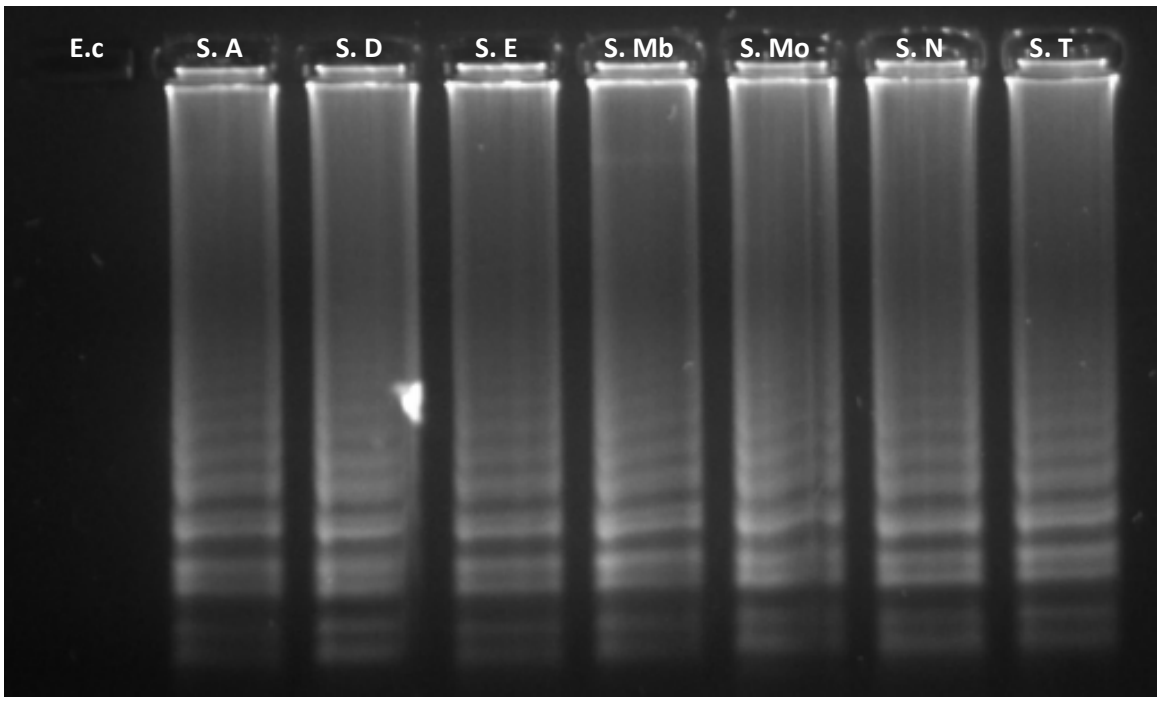

Figure 5.23: Agarose gel showing products of orgA1 LAMP assay amplified for 30 minutes at 65°C targeting multiple *Salmonella* serovars. NT assays showed no product.

Key: E.c = negative control assay using *E. coli* DNA as a template, S. A = *S*. Agama genomic DNA, S. D = *S.* Dublin genomic DNA, S. E = *S.* Enteritidis, S. Mb = *S*. Mbandaka genomic DNA, S. Mo = *S.* Montevideo genomic DNA, S. T = *S.* Typhimurium genomic DNA

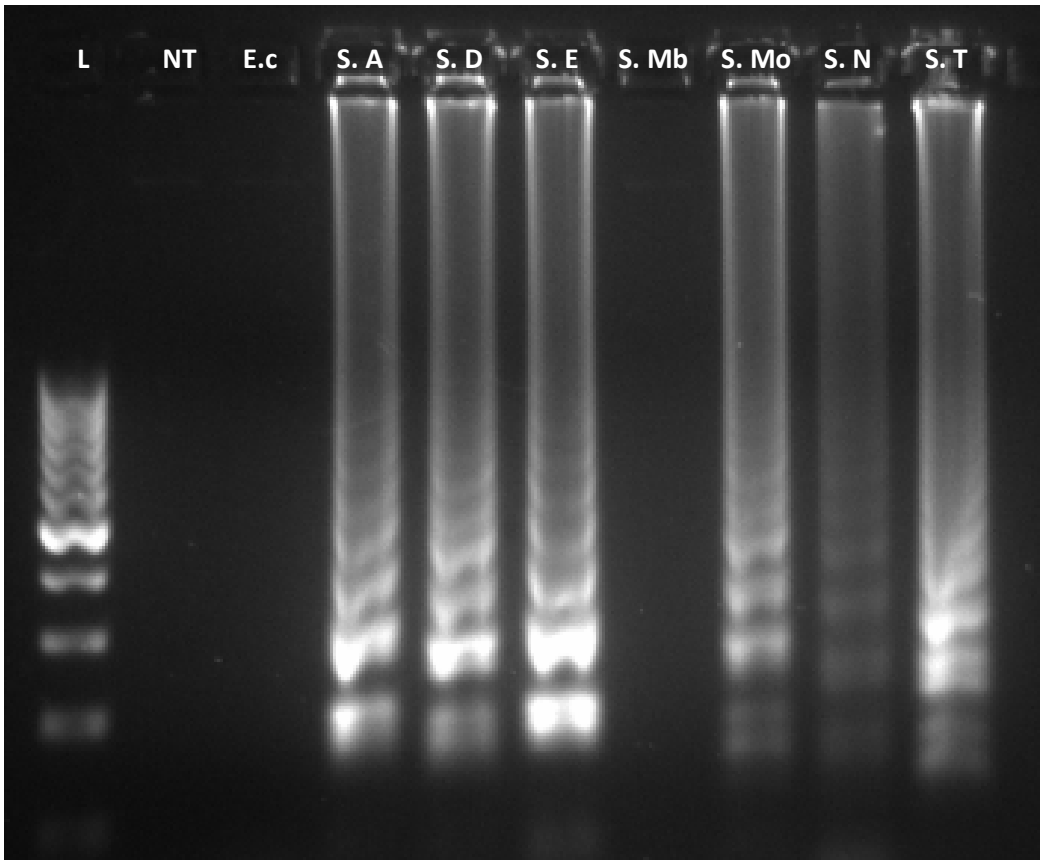

Figure 5.24: Agarose gel showing products of orgA2 LAMP assay amplified for 30 minutes at 65°C targeting multiple *Salmonella* serovars

Key: E.c = negative control assay using *E. coli* DNA as a template, S. A = *S*. Agama genomic DNA, S. D = *S.* Dublin genomic DNA, S. E = *S.* Enteritidis, S. Mb = *S*. Mbandaka genomic DNA, S. Mo = *S.* Montevideo genomic DNA, S. T = *S.* Typhimurium genomic DNA

5.3.8 Contamination with LAMP amplicon from previous amplifications

To remove crossover contamination from previous LAMP assays, workspace and LAMP assays were treated as described in section 5.2.4. Crossover contamination with LAMP amplicon from previous experiments resulted in gels with ladder patterns in all loaded wells (Figure 5.25) and with fluorescence in all reaction tubes (Figure 5.26).

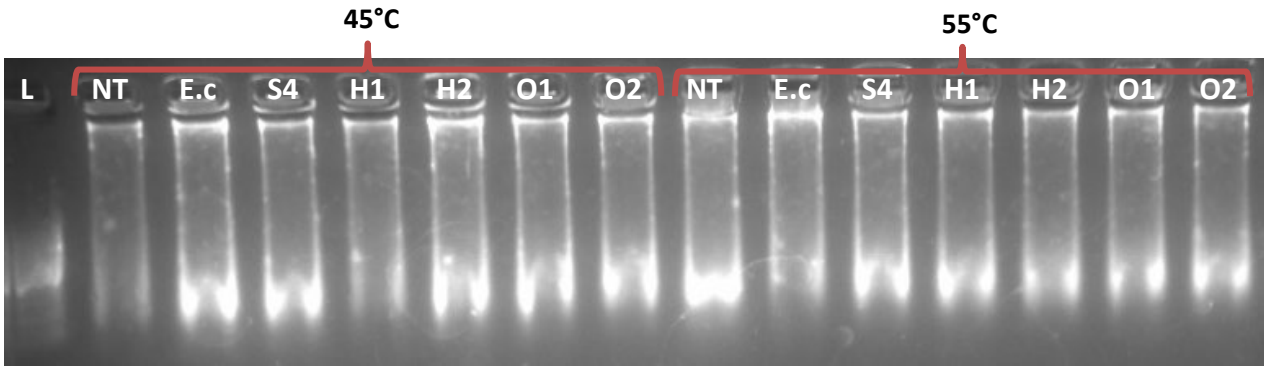

Figure 5.25: Agarose gel showing the products of Fluorometric LAMP assay, amplified for 30 minutes at two different temperatures, targeting *S*. Dublin - this is an example of LAMP amplicon contamination, with no differentiation between negative controls positive controls, or test samples visible.

Key: L = DNA Ladder, NT = No template control with sterile water in place of template DNA, E.c = negative control assay using *E. coli* DNA as a template, S4 = positive control primer set, B2.1 = bapA2.1 primer set, H1 = hilA1 primer set, H2 = hilA2 primer set, O1 = orgA1 primer set, O2 = orgA2primer set

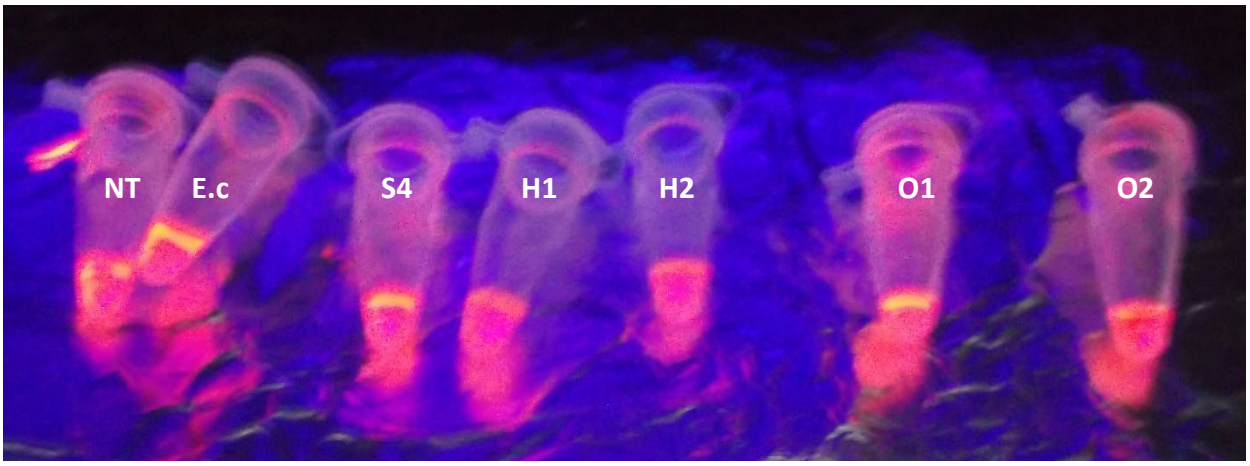

Figure 5.26: Temperature optimisation LAMP reaction tubes corresponding with electrophoresis results in 5.25, targeting *S*. Dublin at 45°C, with propidium iodide used to visualise after termination. This is an example of LAMP amplicon contamination within LAMP assay reaction tubes under UV light, with no differentiation between negative controls, positive controls, or test samples visible.

5.4 Discussion

Within clinical laboratories, amplification-based techniques are used routinely to detect organisms that grow poorly in conventional culture media (Aslanzadeh, 2004). Whilst PCR requires specialised detection devices, extensive DNA purification and extended amplification times, loop-mediated amplification (LAMP) is rapid, simple, and can amplify a few copies of DNA to a tremendous amount in under an hour (Tomita *et al.*, 2008).

Parida *et al*. (2008) noted that the typical electrophoresis pattern of LAMP is a ladder pattern due to continuous amplification forming various sized strands of alternately inverted repeats of the target DNA, this was seen within the electrophoresis gels after the LAMP assays within this study, for example those seen in Figure 5.21.

To facilitate application of LAMP in the field, Parida *et al*. (2008) noted that amplification can be monitored using visual turbidity. Mori *et al.* (2001) detected target DNA using turbidity of magnesium phosphate, a precipitate of the LAMP assay, stating that LAMP conducts amplification and detection in one-step without the use of any detection reagents. As the nucleic acid is amplified, the turbidity derived from the precipitate (magnesium pyrophosphate) is produced according to progress of the reaction and thus can determine whether the targeted DNA is present in specimens (Figure 5.27 - Mori *et al*., 2001).

[1] $(DNA)_{n-1} + dNTP \rightarrow (DNA)_n + P_2O_7^{4-}$

[2] $P_2O_7^{4-} + 2Mg^{2+} \rightarrow Mg_2P_2O_7$

Figure 5.27: Reaction that produces turbidity within LAMP reactions (Mori *et al*., 2001).

When observing turbidity within this study, DNA was initially used at a concentration of 0.1ng/µl, above the calculated detection limit of 0.01ng/µl, however after repeats no turbidity was observed.

To test whether this was due to the sensitivity of the turbidimetric protocol, *Salmonella* sp. DNA was used at 1ng/µl. Turbidity was still not observed, despite incubation within a shaking incubator to aid precipitation, thus the assay tubes were terminated at 60mins and burst spun at 6000rpm in a microcentrifuge. After centrifugation, small loose pellets could be observed but were subjective and difficult to determine.

Mori *et al*. (2001) centrifuged tubes at 6000rpm before observing precipitate at the bottom of the tube which could be confirmed with the naked eye. As the aim of this study is to generate a pen-side *Salmonella* sp. detection LAMP assay, use of a microcentrifuge, a sensitive and relatively expensive piece of laboratory equipment, was deemed as unfeasible in a pen-side setting. However, turbidimetric reading of results would be useful for lab-based LAMP assays, as it allows for closed tube reactions without the need for additional reagents as indicators, as well as quantitative real-time analysis of LAMP turbidity (Mori *et al*., 2001). There are commercially available turbidity readers, that monitor reaction assay in real-time to generate results, unfortunately these systems are often expensive and not applicable for use in low-resource settings.

To enable visualisation of *Salmonella* sp. positive assays, colorimetric dyes were tested. Two types of dye were studied, a metal ion indicator, Hydroxy naphthol blue, and DNA intercalating dyes; Methylene Blue, Nile Blue A, and propidium iodide. When researching dyes appropriate for indicating loop-mediated isothermal amplification, emphasis was placed on the relative expense of the dyes as well as a lack of toxicity and interference with the assay.

Hydroxy naphthol blue, the only dye tested that does not intercalate with DNA, is a metallochromic indicator that forms weak complexes with the magnesium ions within the reagent mix (Goto *et al*., 2009). The dye is displaced as the magnesium ions react within amplification (see figure 5.26), causing a change in colour, indicating the presence of the target DNA (Goto *et al*., 2009).

Intercalating dyes, such as Methylene blue, propidium iodide and Nile blue A, bond with DNA. Nile blue contains aromatic rings that form hydrophobic interactions with base stacks of DNA, as well as positively charged ammonium groups that are attracted to the negatively charged phosphate back bone of DNA (Yang *et al.,* 2000). Yang *et al.* (2000) found that Nile Blue A was safe and convenient when compared to ethidium bromide, in the detection of DNA in gel electrophoresis, without the need of UV light for visualisation. In addition to being non-toxic, Yang *et al.* (2000) found that Nile blue A was tolerant to a wide range of pH (pH 3 to pH 10).

Originally, dyes were added with the reaction mixture before amplification to reduce contamination and increase protocol ease. However, it was soon noted that the intercalating dyes inhibited amplification as they bonded with target DNA. Intercalating dyes were then added at the endpoint of amplification to enable visualisation.

With methylene blue, no obvious colour change was seen when the dye was added to the reagent mix before amplification. Methylene blue intercalates with DNA, thus should have been added after amplification was complete: in future work, this dye should be tested as an end-point indicator for LAMP (Rohs *et al*., 2000).

With Hydroxy naphthol blue, an oversight was made, in that it was assumed that violet was the end point colour, thus when the blue dye was added to the reagent mixture and turned violet it was assumed that reagent mixture rendered the dye useless. However, in hindsight and a greater understanding the mechanisms behind hydroxy naphthol blue within LAMP reactions, hydroxy napthol blue is a strong candidate to enable visualisation of LAMP endpoints.

Goto *et al*. (2009) reported success with hydroxy naphthol blue within LAMP, finding that the dye could indicate a positive reaction to 160 copies/tube, equivalent to that of SYBR green and 10 times more sensitive than calcein. Due to this detection sensitivity, Goto *et al*. (2009) stated that hydroxy naphthol blue was superior to the fluorescent dyes tested, due to easy judgment of positive/negative

results by the naked eye and as well as enabling closed-tube reactions, reducing cross-contamination risks. Due to the ease of use, superior sensitivity and speed, low contamination risk, and lack of specialised equipment, Goto *et al*. (2009) propose that the colorimetric LAMP assay is suitable for clinical diagnoses of many infectious diseases. Future work would look at developing a closed-tube LAMP assay utilising hydroxyl naphthol blue.

With Nile blue, a visual change in colour between negative and positive controls was seen at 45mins, however this was not a strikingly obvious colour change, which was not definitive by the naked eye. Although amplification was visible on electrophoresis gel for assays terminated at time-points <45mins, no visualisation of colour change could be seen with Nile blue.

With propidium iodide, a visual change could be seen between negative and positive controls at 45mins. The dusky pink colour of propidium iodide in the reaction mixture became a lighter brighter pink in the positive controls, however the visualisation could still be considered subjective. As with Nile Blue, despite no change in colour, amplification was visible on electrophoresis gel for assays terminated at time-points <45mins. Potentially, due to a shorter amplification time, less amplicon was produced, thus propidium iodide had less to intercalate and with, resulting in no discernible colour change.

Potentially, the colorimetric LAMP protocol could be improved by increasing the concentration of the dyes, or by testing at greater DNA concentrations to allow for increased amplification. However, as LAMP is known for high sensitivity, a highly sensitive dye was sought, thus a fluorometric protocol was generated testing propidium iodide, SYBR Safe, and SYBR green I.

Mori *et al*. (2001) argue that fluorescent intercalating dyes commonly employed in DNA detection are un-environmentally friendly and highly toxic, which while true for reagents such as ethidium bromide, is not true for SYBR dyes nor propidium iodide. Parida *et al*. (2008) note that to enable field application of LAMP, visual fluorescence could be employed with use of an intercalating dye and a UV lamp.

SYBR green I and SYBR Safe not only intercalate with DNA, but they also bind to the minor grooves of a DNA helix. SYBR Green I binds to the minor groove of DNA and intercalates to base stacks with aromatic ring groups, via van der Waals bonds, with an AT base pair preference (Dragan *et al*., 2012; Zipper *et al*., 2004). Also positively charged thiazole groups bond to the negatively charged phosphates of the DNA backbone (Dragan *et al*., 2012; Zipper *et al*., 2004).

For the fluorometric assays, all dyes were added after termination of amplification and then viewed under a UV light. No fluorescence was seen in negative controls. Under UV light positive controls containing propidium iodide (PI) showed clear bright pink fluorescence. With SYBR safe (SS), yellow/light orange fluorescence could be seen between positive assays whilst negative controls appeared a dull red/orange. SYBR Green I showed bright green fluorescence within positive controls, whilst negative controls remained clear.

All fluorescent dyes showed a clear visual difference between positive and negative assays under UV light. However, SYBR Green I was excluded from further testing due to the comparative expense of the dye. Variances in fluorescence brightness were seen, especially with SYBR safe (Figure 5.11), however this was not quantifiable within the current method. To quantify fluorescence, and colorimetric changes the LAMP assay could be adapted to a 96-well plate and measured within a fluorimeter.

Francois *et al.* (2011) noted that LAMP has a tolerance of various elongation periods: to decrease the protocol time of the fluorescent LAMP assay, reduction in amplification times were tested with the two fluorescent dyes as indicators. With SS, the positive control always showed a strong response at all time points, however with PI until 30mins, a medium response was seen, suggesting that for the Sal4 set, SS is a better fluorescent indicator of amplification than PI. However, with sets H2, O1, and O2, PI showed a medium response at 15mins, whilst SS showed no response at this time point. With set B2.1 both dyes showed a strong response from 20mins. With primer sets B1.1, B1.2, B2.2 and H1,

the response from these sets were sporadic and weak, with fluorescence for SS often only occurring at 30mins. Due to this, primer sets B1.1, B1.2, B2.2 and H1 were not used going forward.

To test the robustness of the LAMP assay to temperature, the fluorometric assays were tested at a range of temperatures, from 25-85°C. When looking at the electrophoresis gels for the temperature ranges for B2.1, amplification can be seen from 45-75°C, however a strong fluorescence response can only be observed at 65°C, suggesting that 65°C is the optimum temperature for the amplification of this primer set. The more amplicon generated, the stronger the fluorescence response. Primer set H2 responded best at 55°C, whilst O1 responded best between 55°C-65°C. The response from O2 was weak across 45°C to 75°C. This suggests that whilst O2, recognises and amplifies *Salmonella* sp. DNA, it does not do so at the same rate as the other primer sets. With the strongest fluorescence response seen around 65°C, these results align with the findings of Francois *et al.* (2011), who found optimal LAMP results between 57-67°C. Francois *et al.* (2011) found that outside this range detection sensitivity declined and at 48°C, amplification was never obtained despite a 60min incubation. As heating elements are cheap and commercially available, in low resource settings at low cost, low energy heating system such as a water bath could be constructed with minimal effort.

Differences in ladder product patterns were seen, with Sal4 and bapA2.1 primer sets often producing visibly brighter bands. Whilst unquantified, differences in band brightness is often relative to the amount of product within the reaction, inferring that the more product the more amplification is occurring. With LAMP, the more specific the primer set, the better the amplification, the greater the amplicon generated, potentially suggesting that bapA2.1 is the most specific primer set generated within this study.

When tested against a panel of *Salmonella* serovars, orgA2 recognised all serovars, except *S.* Mbandaka. This could potentially be due a difference in sequence between the primer set target and the genomic DNA. *S.* Mbandaka complete genome was not available on NCBI database at the time of LAMP primer generation, thus base pair differences could have been missed despite the conserved

nature of *orgA* in other serovars. As of March 2017, a complete genome screening of *S*. Mbandaka (ATCC: 51958) was made available, future work would look at aligning this genome with the *orgA* gene and the orgA2 LAMP primer set to see if it can be determined as to why orgA2 set does not detect *S.* Mbandaka experimentally. However, bapA2.1 and orgA1 detected all 7 of the tested strains. Future work should include testing these primer sets against an increased screen of *Salmonella* sp., nevertheless this study has produced two highly specific pan-*Salmonella* LAMP primer sets, bapA2.1 & orgA1, that can detect *Salmonella* DNA at 65°C in 35 minutes.

Unfortunately, whilst the testing the robustness of the LAMP assay, a contamination issue occurred. Due to the prolific nature of LAMP in the presence of target DNA, cross-over contamination with LAMP amplicon occurred, resulting in negative controls showing amplification when they had not previously.

When developing a LAMP assay, Wang *et al*. (2015) found that primer dimers could cause non-specific amplification, leading to false positives. In the case of this data, this was ruled out as a contamination issue, due to prior success with clear negative controls as well extensive care when designing the LAMP primer sets to avoid dimers.

Despite the wide acceptance of PCR and other amplification techniques, contamination of samples with DNA is a major problem in microbiology laboratories (Borst *et al.*, 2004). Contamination within nucleic amplification techniques could be sourced from reagents, laboratory disposables, or equipment, as well as neighbouring labs, colleagues, PPE and the environments (Borst *et al.*, 2004). However, with LAMP, the most commonly reported contamination issue is cross-over contamination (Borst *et al.*, 2004; Aslanzadeh, 2004; Tomita *et al.*, 2008; Parida *et al*., 2008; Hsieh *et al*., 2014; Saffie *et al.*, 2014; Wang *et al*., 2015).

LAMP is reported as sensitive enough to detect as few as six copies of DNA and amplify these few copies to a very large amount in less than an hour (Notomi *et al.,* 2000; Tomita *et al.*, 2008). It has been shown that LAMP synthesises 10-20µg of specific DNA for 25µl of reaction mixture in 30-60mins,

which is significantly higher than comparable DNA amplification techniques, making LAMP an extremely sensitive reaction (Mori *et al*., 2001; Tomita *et al.*, 2008). Carryover contamination occurs when DNA fragments from previous experiments are re-amplified leading to false positives (Borst *et al.*, 2004; Tomita *et al.*, 2008).

Hsieh *et al*. (2014) highlighted the danger of carryover contamination in LAMP reactions, finding that as little a 1 attogram lead to amplification in LAMP assays. Carryover contaminants were calculated in mass over copy number, due to LAMP generating amplicons of various lengths. An aerosol droplet with a diameter of 0.2µm ($4x10^{-18}$L) was enough to contaminate new assays. Stafford and Ettinger (1972) noted that particles of this size could pass through filters, dependant on continued loading, loading velocity, and the type of filter, thus fibrous pipette tips will not block contaminating LAMP amplicons from transfer via pipette. By avoiding continued loading, using a slow pipette speed, and operating in low temperatures, the effectiveness of filter tips could be improved: if readily available, filter tips with membrane over fibrous filters, thicker filters, or filters with a high adhesion energy would reduce aerosol contamination (Wang and Kasper, 1991; Stafford and Ettinger, 1972).

To prevent carryover contamination, Aslanzadeh (2004) suggests mechanical barriers in the form of separation of preparation, amplification, and analysation areas of the assay, with each site preferably being physically separated and each with its own set of necessary equipment; such as Lab coats, instruments, aerosol-free pipettes and ventilation systems, and reagents. It is also suggested that amplicons can be present on hair, glasses, jewellery, and clothing, when moving from a contaminated room to a clean room (Aslanzadeh, 2004). Borst *et al.* (2004) recommends reagent aliquots are prepared in an area free of nucleic acids, that different freezers and fridges are used in the storage of reagents and samples, each work area should have separate supplies and equipment to substantially reduce the risk of carryover contamination, as well as regular cleaning of anything touched by hands.

Wang *et al*. (2015) noted that non-specific amplification was a limiting factor in the applicability of LAMP when they had contamination whilst developing a LAMP assay, suggesting it could be caused by

slight aerosol pollution from LAMP amplicons. Tomita *et al.* (2008) noted a very high risk of contamination when handling LAMP-amplified product, suggesting that opening and closing of the reaction tube should be conducted in a different room from where reagents and reaction mixtures are prepared.

Handling of waste containing RNA/DNA has been linked to crossover contamination (Borst *et al.*, 2004). Porter-Jordan and Garret (1990) showed that autoclaved amplification products can result in amplification in new assays, thus LAMP assays should not be autoclaved for disposal (Tomita *et al.*, 2008). After observation, reaction tubes should remain closed where possible and kept in double plastic bags that can be incinerated or sealed for disposal, to prevent amplified products from dispersing (Tomita *et al.*, 2008).

Borst *et al.* (2004) noted that negative controls should be run with each assay, matching test reactions with all but the DNA template to monitor contamination, a practice that was followed from the outset of this study.

When eliminating DNA contaminants, the use of irradiation, enzymatic treatment or the use of a corrosive cleaning agent, such as hydrochloride or sodium hypochlorite is often cited (Borst *et al.*, 2004).

Ultraviolet (UV) irradiation oxidises bases, causes single and double strand breaks, and causes the formation of cyclobutene rings between pyrimidine bases within DNA: Borst *et al.* (2004) notes that UV irradiation should be an additional precaution to careful laboratory practice, not a replacement for it. Aslanzadeh (2004) suggests that when not in use pipettes and other devices should be stored in a UV light box.

The efficacy of UV to eliminate DNA depends on the size of the sequence, the time of treatment, the distance of the light (Pauda *et al*., 1999). Greater distances from the UV light reduced efficiency and more time was needed to eliminate larger sequences (Pauda *et al*., 1999). Within reaction mixtures

containing dNTPs, up to 90mins of UV treatment at 10cm is required to eliminate PCR amplicons (Pauda *et al*., 1999). Pauda *et al*. (1999) noted that by combining UV light treatment at 10cm to reaction mixtures in cabinets with a downward flow of air allowed them to complete thousands of contamination free PCR assays. Cabinets with a downward flow of air, ensured that aerosols were pushed down to the base of the cabinet, away from the top of reaction tubes (Pauda *et al*., 1999). Whilst UV light is inexpensive, it is not as effective against GC rich DNA sequences or short (<300bp) amplicons (Aslanzadeh, 2004).

Aslanzadeh (2004) states that all workstations should be cleaned with 10% sodium hypochlorite solution, followed by removal with ethanol. Sodium hypochlorite (bleach) breaks the hydrogen bonds between DNA base pairs denaturing the DNA, as well as causing alkaline cleavage of the phosphate backbone.

Hsieh *et al*. (2014) developed an integration of LAMP with uracil-DNA-glycosylase (UDG) digestion in an all-in-one closed tube reaction to eliminate false-positives from carryover contaminates. By using uracil to replace thymine in the dNTPs used to generate LAMP amplicons, carryover contamination can be degraded by the enzyme UDG in new reactions, leaving target DNA intact. At the temperature of the LAMP reaction (65°C), UDG is denatured, allowing the formation of new amplicons in the presence of the target DNA, by *Bst* 2.0 DNA polymerase that incorporates dUTP. Using UDG-LAMP, Hsieh *et al*. (2014) showed the method can significantly reduce false-positive results due to carryover contaminants, with a limit of detection of 10 attograms of carryover contaminant present in 4000 copies of target DNA. However, UDG works best with thymine rich amplification products, showing a reduced activity with GC rich targets, and is relatively expensive (Aslanzadeh, 2004). Also, Hsieh *et al*. (2014) did observe some inhibition of LAMP in the presence of UDG and suggested this could be improved by adjusting doses, exploring UDG enzymes more susceptible to thermal deactivation, or by adding an intermediate UDG-inactivation step.

Saffie *et al.* (2014) noted that the addition of SYBR green I to the reaction mix before heating would inhibit the LAMP reaction, so they added the dye once the reaction was completed. Due to opening the tube after amplification, they suffered cross-contamination, a major challenge in developing their in-house LAMP assay. Saffie *et al.* (2014) decided to use calcein instead, as it would not inhibit the reaction when added before amplification, removing the need to open tubes and risk contamination, and was cheaper. Reaction tubes were reviewed under a battery operated hand-held transistorised UV lamp. Hsieh *et al*. (2014) used calcein within a one-pot, closed vessel reaction, noting that in the case of amplification, strong calcein fluorescence was directly observable under ambient light, showing an orange to yellow colour change, or UV irradiation.

Calcein in the reaction mixture initially combines with manganese ion to remain quenched, when amplification occurs, the manganese ion is stripped from calcein by the pyrophosphate ions that are produced as a by-product from the reaction substrate from deoxyribonucleotide triphosphates (dNTPs) (Tomita *et al.*, 2008; Parida *et al*., 2008). This results in fluorescence emission from calcein, which combines with magnesium ions in the reaction mixture, increasing the fluorescence (Tomita *et al.*, 2008; Parida *et al*., 2008). The presence of fluorescence can indicate the presence of target DNA without opening the tube, preventing carry-over contamination from post-amplification products (Parida *et al*., 2008).

Whilst Parida *et al*. (2008) suggest that opening the reaction tube after amplification should generally be avoided to prevent carry-over contamination with the post-amplification products, they also discuss adding intercalating dyes after assay termination. Before issues with contamination, the scope of damage caused by cross-over contamination was not fully understood, papers discussing developing LAMP assays do not emphasise the issues faced when contamination occurs due to the prolific action of LAMP (Saffie *et al*., 2014).

To prevent carryover contamination, barriers must be in place prior to when the amplification technique is used initially (Aslanzadeh, 2004). Hsieh *et al*. (2014) stated that there are currently no

effective means for eliminating LAMP carryover contamination, leaving laboratories using LAMP to rely on careful preventative methods that are prone to failure. Sometimes requiring an assay re-design, once contamination occurs, the process of decontamination is costly and time-consuming (Hsieh *et al*., 2014).

Francois *et al.* (2011) added their LAMP reagents in a cold rack (4°C) and required <5min when performed by an experienced technician. To test the effect of sample preparation procedure, samples were kept at room temperature for up 30mins before incubation. At room temperature (22°C), no effect on efficacy was observed, however at 37°C false-positive results were generated in control samples. Incubation for short periods of time at 37°C or longer periods of incubation at 20°C did not yield false-positive results as observed within the qPCR assays. Throughout the study, reagents were added at room temperature. When testing visualisation methods, reagent addition took ≤5min, however once a visualisation protocol had been established, the amount of assay tubes was bulked, taking ≤30min of preparation before amplification.

The contamination within this study coincided with the increase in the amount of LAMP assays being generated, as well as an unusually hot summer increasing the ambient temperature of the laboratory greatly. Whilst an extensive decontamination protocol was initiated, all assays were still showing amplification and further research into this issue was undertaken. Waste was being autoclaved, including LAMP assay waste, within the laboratory experiments were occurring in which as noted above can be a cause for contamination. Despite careful practice, barriers to carryover contamination were not in place before initial use of the amplification technique and the optimised assay required opening the amplified LAMP assay to enable visualisation. It is encouraged, that when developing a LAMP assay, a thorough search into carryover contamination is undergone, as well as methods into closed tube LAMP assays to avoid the issues faced within this study. Additionally, whilst the techniques are applicable within a laboratory setting, they may not be always be practical, for example in third world countries or whilst on farm.

Therefore, future work would look at integrating UDG digestion, as suggested by Hsieh *et al*. (2014), into the current optimised protocol to remove current LAMP amplicon cross-over contamination, with a view to developing an all-in-one closed tube reaction assay, utilising calcein or hydroxy naphthol blue for visualisation, using the two promising LAMP primer sets generated within this study. This would allow for completion of the robustness testing planned, which due to time constraints were not completed, whilst maintaining the target of generating a simple, reliable assay for pen-side use in low-resource settings.

To test the robustness of the assay further, experiments to determine the sensitivity of the LAMP assay would be undergone using diminishing concentrations of template DNA, including the effect decreasing concentrations of target DNA may have on visualisation using dyes. It would be interesting to dilute dye concentrations, to further reduce the cost of the assay without losing sensitivity or ease of visualisation. Additionally, testing the robustness of the assay through calf scour and monitoring any changes to pH could have on the sensitivity and specificity of the assay.

Wilcox *et al*. (2013) noted that, along with designing specific primers, the best solution for testing the specificity of an assay is to challenge assays with pure and mixed samples of target and non-target DNA. Ideally this would be completed whilst looking at the effect of calf scour on the efficacy of the LAMP assay. Kaneko *et al*. (2006) evaluated the tolerance of LAMP and PCR against biological substances, finding that LAMP had a higher tolerance to the substances tested, including serum, plasma and urine whilst Francois *et al.* (2011) noted that LAMP performed well despite the presence of untreated faecal matter. Francois *et al.* (2011) determined that adding a 1:25 amount of urine or stool sample had no effect on amplification efficiency of 1000 gene copies.

LAMP is a practical technique for low resource settings, as the only equipment needed is a heat block/water bath that can maintain a temperature of 65°C. Saffie *et al.* (2014) note that LAMP is a promising and reliable method for resource -limited settings; they used a compact, portable heating block that can be used wherever 12V power was available but suggested point-of-care testing could

be achieved by using a rechargeable heating block, thermostabilised reagents and simplified DNA extraction. Mori *et al*. (2001) noted that LAMP has the potential to not need electrical connections suggesting that natural energy could be used as a power source to furnish an isothermal environment.

LAMP is highly robust and sensitive, even when samples are impure (Francois *et al.*, 2011; Parida *et al*., 2008). With simple operation, easy naked eye monitoring and cost-effective reaction equipment, LAMP is easily adaptable for field conditions (Parida *et al*., 2008). These features are particularly useful for testing in clinical settings, especially within developing countries where the need for rapid diagnostics of emerging infections is most urgent (Francois *et al.*, 2011).

Amplification efficiency of LAMP is extremely high as there is no time loss from thermal change, as the reaction is isothermal, running at the optimal temperature of the enzyme (Mori *et al*., 2001). Within 15-60mins LAMP amplifies DNA 10^9-10^{10}-fold, making it highly efficient and has a detection limit of a few copies, being comparable to the limit of PCR (Parida *et al*., 2008; Notomi *et al.,* 2000). Notomi *et al*. (2000) found that LAMP not only had a high efficiency but is not significantly influenced by non-target DNA within the reaction assay.

For LAMP, specificity is extremely high due to the primers targeting six distinct regions of the template DNA, amplifying a specific gene with discrimination down to a single nucleotide difference (Mori *et al*., 2001; Parida *et al*., 2008; Tomita *et al.*, 2008). Due to this the background signals associated with all nucleic acid amplification, is greatly reduced (Notomi *et al.,* 2000).

With LAMP there is no need for heat denaturation double stranded DNA, unlike with PCR, due to the use of a DNA polymerase with strand displacement activity (Saffie *et al.*, 2014; Parida *et al*., 2008; Nagamine *et al*., 2002). Not only does this negate the need for expensive thermal cycling equipment, but after mixing reagents, amplification and detection can be carried out in a single step, at the optimum temperature of the DNA polymerase greatly reducing amplification time (Parida *et al*., 2008; Notomi *et al.,* 2000). The isothermal nature of LAMP combined with cyclic amplification that is greatly

accelerated by the incorporation of loop primers makes LAMP a quick detection technique (Nagamine *et al*., 2002).

Saffie *et al.* (2014) generated an in-house LAMP assay to detect *Salmonella enterica* serovar Typhi and Paratyphi, noting that LAMP needed minimal instrumentation, had a short reaction time (~60mins), and allowed for easy visual analysis. They reported 100% specificity and sensitivity, as compared to culture, and noted that, whilst the comparison PCR assay was as sensitive as their in-house LAMP assay, LAMP was a better diagnostic alternative due to short reaction time and not requiring an expensive thermal cycler. Additionally, Francois *et al.* (2011) noted that the resistance of LAMP to prolonged warming of the mastermix is a critical difference from conventional PCR assays, which require cold blocks for the preparation of reaction mixtures or the use of Uracil-DNA-Glycosylase to avoid false-positives.

Isothermal amplification lends itself nicely to pen-side detection and as does the robustness of LAMP to biological samples, such as scour. With high specificity and sensitivity, and a robustness not often associated with molecular detection, LAMP has proved to be as a simple, quick assay for DNA detection. This work has shown that *bapA, hilA* and *orgA* can be targeted to detect *S.* Dublin, and that the bapA2.1 and orgA1 LAMP primer sets generated with bioinformatic techniques can be used to detect multiple *Salmonella* serovars in 35 minutes without the need for expensive apparatus.

6. Immunoassays

6.1 Introduction to immunoassays

When infected with *Salmonella* sp. an animal's immune system recognises various antigen on the bacteria and produce antibodies to bind and respond to the infection (Ewald *et al*., 2013). Antibodies are capable of recognising antigens with high specificity: there are two main types, monoclonal antibodies and polyclonal antibodies (Felix and Angnes, 2018). Monoclonal antibodies can recognise a single reactive region (epitope) on the target pathogen and are more specific with a reduced chance of cross-reactivity (Felix and Angnes, 2018). Polyclonal antibodies recognise multiple epitopes, potentially on multiple species of microbe, making them less specific and more likely to cross-react (Felix and Angnes, 2018). Antibodies are used in immunoassays for pathogen detection by utilising the high specificity of the antibody-antigen interaction (Holford *et al*., 2012).

Immunoassay use is widespread; in clinical diagnosis they have been particularly successful (Zhu *et al.*, 2019). Enzyme-Linked Immuno-Sorbent Assays (ELISA) are a well-established clinical immunoassay that are considered the 'gold-standard' in immunoassays (Mobed *et al.,* 2019; Holford *et al*., 2012). As such ELISA are often used as the standard for benchmarking newly developed immunoassays and immunosensors. However, immunoassays can be highly labour intensive, time consuming and expensive: ELISA requires several working, incubation, and washing steps (Ewald *et al*., 2013; Holford *et al.*, 2012).

There are several types of immunoassay, including direct, indirect, and sandwich, which can be used to capture the antigen of the target, or to detect antibodies that recognise the target. In general immunoassays utilise antibody-antigen affinity to capture the target and a detection system to indicate the presence of the target binding (Mobed *et al.,* 2019). The current research looks at detection of *Salmonella* antigen, specifically surface proteins, through use of direct and sandwich immunoassays. When designing an immunoassay, the antibody-antigen reaction is essential to establish good specificity and selectivity (Zhu *et al*., 2019). When selecting antibodies, those targeting

flagella only were screened out, as not all *Salmonella* serovars display H antigens (Table 6.1; McQuiston *et al.,* 2011). Commercial antibodies for the detection of *S.* Dublin were initially sought.

Table 6.1: The O and H antigens for the *Salmonella* serovars used within the immunoassays of this study. O antigens are somatic antigens found in the cell wall of *Salmonella* bacteria. *Salmonella* O groups used to be denoted by letters, however due to there being more O groups than letters, nomenclature has changed to numbers, with the old nomenclature sometimes being displayed in accompanying brackets, eg. O:4 (B). *Salmonella* sp. have phase variation of flagellar (H) antigens. Different motile phenotypes are displayed dependant on the phase of the *Salmonella* sp.: monophasic serovars, with only one phase of H antigen, can be motile or non-motile (adapted from Grimont and Weill, 2007).

Key: underlined = O factors determined by phage conversion. Phage conversion is when a bacteriophage inserts its nucleic acid into a bacterium. The phage DNA is integrated into the host bacterium's genome and can be passed onto daughter cells. [ ] = O/H factors that may be present but not through phage conversion. H factors in square brackets are often only present in wild strains.

***Salmonella* serovar**	**'O' group**		**Somatic (O) antigen**	**Flagellar (H) antigen**	
	Old nomenclature	Current nomenclature		Phase 1	Phase 2
Agama	B	O:4	4,12	i	1,6
Bovismorbificans	C_2	O:8	6,8,20	r,[i]	1,5
Dublin	D	O:9	1,9,12[Vi]	g,p	-
Enteritidis	D	O:9	1,9,12	g,m	-
Mbandaka	C_1	O:7	6,7,14	z_{10}	e,n,z_{15}
Montevideo	C_1	O:7	6,7,14	g,m,[p],s	[1,2,7]
Newport	C_2	O:8	6,8,20	e,h	1,2
Typhimurium	B	O:4	1,4,[5],12	i	1,2

For a direct immunoassay, the target antigen is adhered to the reaction surface and detected by a detection antibody that is conjugated to an enzyme. To determine the amount of antibody-antigen reactions, the substrate for the detection enzyme is added and the resulting reaction is recorded. For a sandwich assay, the capture antibody is adhered to the reaction surface. The target antigen is captured by the antibody if present within the sample. A detection antibody, conjugated to an enzyme, is added and adhere to the captured antigen. The substrate for the conjugated enzyme on the detection antibody is added and the resulting reaction is recorded. For ELISAs, the measurement is colorimetric, the change in colour due to the enzyme-substrate reaction is recorded. For potentiometric biosensors, the change in electric potential is recorded due to enzymatic turnover

resulting in a change in charge due to the loss of electrons (Cork *et al*., 2012; Figure 6.1). Both ELISAs and potentiometric biosensors are used within this study.

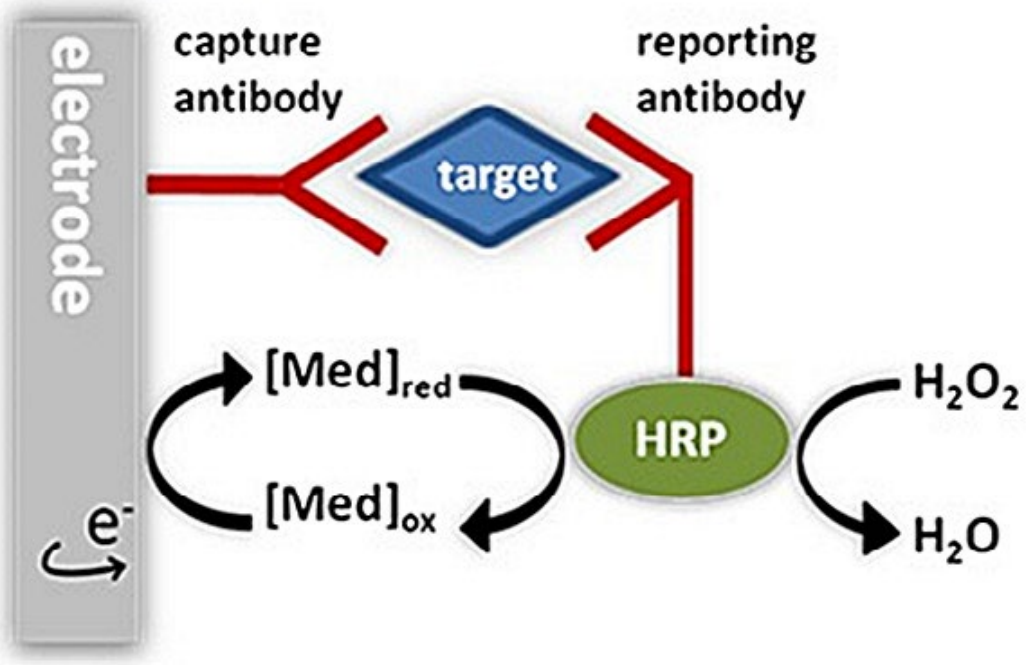

Figure 6.1: A schematic representation, from Kokkinos *et al*. (2016), of the mechanism of a sandwich immunoassay developed upon a potentiometric biosensor utilising Horseradish Peroxidase (HRP) as the detection enzyme. The electrode measures the charge generated from the enzymatic turnover of hydrogen peroxide as the surface of the electrode is depleted of electrons (e^-).

Within this study Horseradish peroxidase (HRP) is used as the conjugated detection enzyme, with 3,3',5,5' – Tetramethylbenzidine (TMB) as the chromogenic substrate. HRP reduces hydrogen peroxide into water, TMB acts as a hydrogen donor for this reaction, resulting in a colour change of clear to blue. To halt this reaction, acid was added resulting in the blue solution turning yellow, the absorbance of which can be read within a spectrophotometer at 450nm.

Cheung and Kam (2012) noted that to significantly reduce the resources required in routine laboratory operation, rapid methods for *Salmonella* detection are needed and would enhance productivity and efficiency of public health laboratories. Additionally, Holford *et al*. (2012) noted that large sample areas, such as fields, require portable, rapid diagnostics that are cheap with great sensitivity. Routine sampling for environmental and public safety purposes is commonplace to detect contamination increases and determine future actions, therefore precision and accuracy are important (Holford *et*

al., 2012). Holford *et al*. (2012) note that screen-printed electrodes, biosensors, are a promising technology to solve the issues commonly associated with immunoassays.

A biosensor is an analytical device that integrates a physicochemical transducer with a biologically derived recognition of molecules, transforming biological interactions into signals that can be measured and recorded (Bahadir and Sezginturk, 2015). In general biosensors produce an electronic or optical signal proportional to the specific interaction between the target and recognition molecule, which has been immobilised on the biosensor, types of molecules can include enzymes, antibodies, phages, aptamers, ssDNA (Bahadir & Sezginturk, 2015). Bahadir and Sezginturk (2015) note that a lack of specific, low-cost, rapid, sensitive, and easy detection for biomolecules has resulted in the development of biosensor technology. The diagnosis and monitoring of diseases can require extensive effort for routine and follow-up tests: often requiring specialised personnel, time, and high sample volumes. In comparison biosensors have many advantages over typical detection methods such as; low cost, high sensitivity, rapid response, low sample volumes, and easy operation without the need for expensive instrumentation or specialised personnel (Bahadir and Sezginturk, 2015). Additionally, portability, miniaturisation and on-site monitoring are often reported as important advantages of biosensors over other analytical techniques (Zhu *et al*., 2019; Felix and Angnes, 2018; Kokkinos *et al*., 2016; Bahadir and Sezginturk, 2015; Konchi *et al*., 2007).

Biosensors have been reported as a highly specific, highly sensitive, rapid, and cheap method for detecting a range of analytes, and commercial biosensors are used across multiple analytical sectors. Examples of which are process control, environmental samples, biological warfare, the food industry (both composition and contaminate detection), and in clinical settings for detecting pathogens such as *E. coli* O157, *H. pylori*, Influenza strains, HIV, *M. tuberculosis* and malaria (Felix and Angnes, 2018; Bahadir and Sezginturk, 2015; Fei *et al.*, 2015; Holford *et al*., 2012).

There are various types of biosensor, one is electrochemical biosensors that measure the change in potential of an assay (Holford *et al*., 2012). Electrochemical biosensors are powerful, versatile tools,

that offer good accuracy and precision with simple instrumentation (Haji-Hashemi *et al*., 2019; Felix and Angnes, 2018; Holford *et al*., 2012). Types of electrochemical sensors can be based on amperometric, impedimetric, or potentiometric transduction (Holford *et al*., 2012).

Felix and Angnes (2018) noted that electrochemical immunosensors have gained prominence recently, due to the advantage of sensitivity, selectivity, low detection limits, portability, and the possibility of simultaneous multi-target analysis (Zhu *et al*., 2019; Haji-Hashemi *et al*., 2019; Kokkinos *et al*., 2016; Bahadir and Sezginturk, 2015). Additionally, due to relative ease of miniaturisation of electrochemical biosensors to hand-held devices, it is suggested that electrochemical biosensors are more suitable for on-site analysis (Zhu *et al*., 2019; Felix and Angnes, 2018; Kokkinos *et al*., 2016; Bahadir and Sezginturk, 2015; Konchi *et al*., 2007). Kokkinos *et al*. (2016) note that electrochemical biosensors are useful due to the low cost of instrumentation, scope for mass fabrication, short response time, and their fabrication simplicity, as screen-printed electrodes can be produced relatively cheaply with existing technology (Holford *et al*., 2012; Konchi *et al*., 2007).

Immunosensors are a type of electrochemical biosensor that are based on antibody-antigen interactions on a transducer surface upon an electrode and combine the advantages of high sensitivity and selectivity with real-time monitoring (Mobed *et al*., 2019; Felix and Angnes, 2018; El Ichi *et al.*, 2014). Low manufacturing costs and miniaturisation combined with very small working amounts of sample and reagents makes immunosensors cost-effective, allowing them to be disposable, which can be an advantage in protocol adoption (Skladal, 2019; Felix and Angnes, 2018; Derkus, 2016). Felix and Angnes (2018) reported immunosensors to be highly specific method that gives rapid, reliable responses and Mobed *et al*. (2019) suggest that immunosensors could take the place of ELISA.

Like ELISAs, immunosensors can be direct, a signal is generated from the antibody-antigen reaction, or indirect, a signal is generated after binding has occurred by activation of an attached label, such as HRP (Holford, *et al*., 2012). The sensitivity of an immunosensor is reported to be strongly connected to the affinity of the antibody to antigen reaction, as well as the properties of the transducer (Zu *et*

al., 2019; Haji-Hashemi *et al*., 2019; Fei *et al*., 2015; Purvis *et al*., 2003). Potentiometric biosensors have been relatively unpopular, due to issues with sensitivity, accuracy, precision, and stability (Purvis *et al.*, 2003). Purvis *et al.* (2003) noted that it was felt that potentiometric sensors would not work due to interference from the sample matrix occluding signals derived from the specific binding of analytes. Purvis *et al.* (2003) developed a potentiometric biosensor called the Universal Transducer System™ (UTS) which is the basis of the Vantix™ Research Tool (VR1 and VR2), which they report to not suffer from the previous issues associated with potentiometric assays, due to a new polymerisation technique that gives an unexpected robustness and sensitivity to the polypyrrole layer of their potentiometric probes.

The Vantix biosensor probes are single use, disposable, and screen-printed. They comprise of conduction tracts, encased in dielectric for insulation, connected to a black carbon test electrode and in integral reference silver/silver chloride electrode (Strand *et al.,* 2011). On the test electrode polypyrrole, an electroconductive polymer, forms the sensing element and immobilisation matrix for the assay reaction (Stead *et al*., 2011; Purvis *et al.*, 2003).

Purvis *et al*. (2003) note assays upon the UTS probes use established ELISA techniques, in which sample analyte is captured and complexed with a secondary antibody labelled with an enzyme. These enzyme-linked immunocomplexes form on the polypyrrole layer of the electrode. This can then be measured by adding substrate appropriate for the enzyme linked to the antibody. The probe measures the change in potential of the electrochemical reactions occurring on or near the test electrode: the change in potential is related to the concentration of the enzyme-linked immunocomplexes and thus the concentration of the sample target (Purvis *et al.*, 2003).

Purvis *et al.* (2003) call the mechanism, by which the probe measures the change in potential, a charge step, wherein the polypyrrole layer of the test electrode undergoes electron depletion due to the electrochemical activity occurring on/near it, causing a shift in potential of the electrode. This change is measure in millivolts (mV) and compared to that of the reference probe, thus developing a signal.

During development and testing, Purvis *et al*. (2003) determine that the UTS exhibits ultra-sensitivity with good precision, reproducibility, stability, and the ability to perform a wide range of immunoassays targeting high and low molecular weight analytes. The UTS technology was developed into the Vantix Research Tool (VR1: Vantix™ Ltd, Cambridge, UK) and commercialised.

Stead *et al*. (2011) developed an assay on the VR1 to detect Tylosin within feed and found the qualitative screening method developed to be sensitive and robust. Tylosin is an antibiotic that has historically been used as a growth promotor within farms, a practice that is now banned within the European Union. Stead *et al.* (2011) determined that the VR1 is a low-cost, high throughput, versatile format, that they were able to develop a screening method of up to 12 samples (including controls) within 45min. They determined the VR1 assay to be reliable and repeatable, with no significant differences between batches of probes. During the study they reported no false compliant or non-compliant results and showed the potential for field-based assays, as well as multiplexing. They highlighted the relative ease of assay development, noting that with good quality, well characterised immunoreagents available, assay development and validation can be completed in under a month, a relatively short amount of time. Additionally, they noted that the Vantix system could be automated, within their validation experiments they utilised a liquid dispensing system.

Cork *et al*. (2012) also developed a biosensor assay using the VR1 to detect bovine herpes virus 1 (BoHV-1) noting that the VR1 probes were flexible and easily handled. They suggest that the VR1 is a promising platform for routine immunological testing as the conversion of established ELISA assays to a more rapid test format was achieved and that the system is robust and ready for field testing, due to the BoHV-1 assay working through serum and undiluted milk samples in concentrations higher than can be used in ELISA.

The BoHV-1 biosensor assay allows for the level of antibodies present within a bulk milk sample to be determined, equivalent to that of the parent ELISA with similar sensitivity, repeatability, and specificity. From this, Cork *et al.* (2012) suggest that the use of Vantix biosensor may be applicable to

animal/human health situations where the correct downstream action, such as treatment or quarantine, needs a quantitative result. Vantix are currently developing a hand-held device incorporating the current technology, offering the potential for more rapid (*circa* 5 min) automated testing, further improving this technology for point-of-care testing (Cork *et al*., 2012).

The Vantix platform has been reported to be simple, practical, and cost-effective (Purvis *et al*., 2003; Stead *et al*., 2011; Cork *et al*., 2012). The Vantix research tool (VR1; Figure 2.2) and Vantix research tool 2 (VR2; Figure 2.4), an optimised version of the original Vantix platform, comprised of a reader, biosensor probes and a stand, the VR2 probes come as a comb of 12, with a clip to secure them to the reader. Without the need for specialist biosensor knowledge, the Vantix platform allows for the adaption of existing and established ELISA protocols, using the same antibodies/antigen and reagents, achieving the same sensitivity and specificity as the parent ELISA (Cork *et al*., 2012; Stead *et al*., 2011; Purvis *et al*., 2003). However, the Vantix immunoassays are rapid when compared to ELISA, with Stead *et al.* (2011) developing an assay totalling 45mins, compared to 4 hours for the parent ELISA assay. Rapid, simple Vantix assays would potentially enable quick and efficient turnaround in diagnostic laboratories, where there is an increasing demand for same-day testing as well as on-site or point-of-care testing (Cork *et al.*, 2012). Additionally, Vantix assays would be useful for testing in environments with limited resources such as on farm or in developing countries, where access to electricity, expensive equipment and trained personnel could be limited (Cork *et al*., 2012).

This study aims to develop an immunoassay using the VR2 for the detection of *Salmonella* sp. through calf scour. To enable this, the following objectives were undertaken;

- Identify commercially available pan-*Salmonella* antibodies & use these antibodies to develop an ELISA to determine optimal immunoassay reagents and conditions
- Adapt the ELISA to an immunoassay upon the VR1/VR2 and optimise to reduce overall protocol time whilst maintaining sensitivity
- Challenge the Vantix immunoassay with calf scour and optimise

6.2 Expanded immunoassay methodology

Antibody storage, dilution, and conjugation method were as described in section 2.6. When selecting antibodies, any that recognised flagella (H antigen) only were screened out, due to some salmonellae being monophasic. For ease of use, Table 2.15 – which describes the antibodies used within this research to detect *Salmonella* sp., has been repeated here.

Table 2.15: The antibodies used within the study, with lab references and relevant information

Antibody	Supplier	Lab reference	Isotype	Raised in	Type	Information on reactivity (summarised from Supplier product info)
Salmonella Antibody (5D12A)	Bio-rad	BMM	IgG1	Mouse	Monoclonal	Broad Reactivity antibody, clone 5D12A recognises the core antigen bearing O antigens. Antibody recognises *Salmonella enterica* serogroups; A (*S*. Paratyphi A), B (*S*. Typhimurium), C1 (*S*. Choleraesuis), C2, (*S*. Newport), D (*S*. Enteriditis), E1 (*S*. Anatum) and E2 (*S*. Selandia). Does not cross-react with *E. coli* 055:B5, *E. coli* K12 or *Klebsiella pneumoniae.*
Salmonella Group Antigen Antibody: HRP	Bio-rad	BRP	IgG	Rabbit	Polyclonal	Antibody is polyvalent for *Salmonella* O and H antigens, is unabsorbed and may cross react with related Enterobacteriaceae.
Salmonella Polyclonal Antibody	Thermofisher	TRP	IgG	Rabbit	Polyclonal	Antibody raised to a mixture of *S*. Enteriditis, *S*. Typhimurium and *S*. Heidelburg and is polyvalent for all "O and H" *Salmonella* antigens.
Salmonella LPS Monoclonal Antibody (A99H)	Thermofisher	A99H	IgG2a	Mouse	Monoclonal	Antibody is specific for common LPS core of all Salmonellae O-serogroups tested; A, B, C1, C2, D, E1, E3, E4, F, G1, G2. Does not cross-react with *E. coli*, *Klebsiella*, *Citrobacter, Pseudomonas, Yersinia, Shigella, Proteus* or *Legionella.*

Unless otherwise stated, all immunoassays were run in triplicate. Preparation of the buffers and substrates used within the immunoassays were as stated in section 2.7. ELISA assays were completed as described in section 2.8 and potentiometric immunoassays using the Vantix System were completed as described in section 2.9

6.2.1 Colony counts to determine the average number of bacteria in a range of absorptions at 600nm.

To determine the average number of bacteria at a range of absorptions, cultures of *E. coli, S.* Dublin, and *S.* Mbandaka, were grown aerobically in 100ml nutrient broth within conical flasks on a shaking incubator at 37°C for 16-18 hours. Within 50ml polypropylene centrifuge tubes, 20ml of the cultures were centrifuged at 5000rpm for 20mins to harvest the cells. The resultant pellets were then washed in 10ml carbonate bicarbonate buffer, vortexed and then centrifuged for 20mins, 3 times. Pellets were then re-suspended in 10ml carbonate bicarbonate buffer and a stock solution at an absorption of 1.0 at 600nm was prepared using a spectrophotometer, with carbonate bicarbonate buffer as a blank.

Using a 96 well plate (Nunclon, flat-bottomed), 50µl of carbonate bicarbonate buffer was added to all wells within row 1 except the well in column 1. In column 1, 100µl of bacteria was added to first well. A 1:2 serial dilution was completed down to 1/128 the concentration of bacteria. A nutrient agar plate (NA – Oxoid, CM0309) was split into 6 sections. In the first section 10µl from the sixth well in column 1 was dispensed, in the second section 10µl from the fifth well in column 1 was dispensed, repeating this process until in the sixth section 10µl from the first well in column 1 was dispensed. Using a sterile cotton bud, working from lowest concentration to highest, the aliquots were spread within the bounds of their section. This process was repeated, on a separate NA plate, for each column within the 96well plate. Plates were left to dry for a minimum of 5 mins, then incubated aerobically at 37°C for 16-18hrs. Colonies were then counted: working from the highest concentration, the first dilution that allowed for between 30-300 colonies to be counted within its section was counted. Colony forming units (CFU/ml) were determined using the following formula: CFU/ml = (No. of colonies x dilution factor) / volume loaded onto NA plate (ml).

6.3 Immunoassay Results

To determine the average amount of bacteria at a range of optical densities when measured at 600nm, colony counts were performed and colony forming units were calculated, see Table 6.2 (for methods, see section 6.2.1).

Table 6.2: The average amount of bacteria in range of dilutions from a stock solution of bacterial suspension of 1 OD units at 600nm (n=3).

Dilution factor from an absorption of 1 at 600nm	**Average number of bacteria (cfu/ml)**		
	E. coli	*S.* Dublin	*S*. Mbandaka
1	$1.50x10^8$	$3.36x10^8$	$1.71x10^8$
1/2	$5.80x10^7$	$9.23x10^7$	$8.00x10^7$
1/4	$5.72x10^7$	$8.95x10^7$	$4.74x10^7$
1/8	$1.36x10^7$	$4.07x10^7$	$2.63x10^7$
1/16	$1.15x10^7$	$2.13x10^7$	$1.88x10^7$
1/32	$4.15x10^6$	$8.67x10^6$	$1.51x10^7$
1/64	$2.20x10^6$	$8.10x10^6$	$8.35x10^6$
1/128	$1.02x10^6$	$2.07x10^6$	$1.74x10^6$

6.3.1 ELISA for the detection of *Salmonella* sp.

ELISA assays were used to determine the binding activity of the antibodies in table 2.15 to various *Salmonella* serovars. Several ELISA protocol steps were optimised to improve the efficacy of the developed assay. Error bars represent the standard deviation within the data set.

6.3.1.1 Direct ELISA and adsorption of antibodies to limit E. coli binding activity

Direct ELISA assays were used to confirm that the commercial antibodies recognised various *Salmonella* strains specifically. Figure 6.2 shows that all three antibodies produce a greater signal for the *Salmonella* strains than for *E. coli*. It can also be observed that there is a large deviation between data sets.

To reduce cross-reactivity of *E. coli*, the antibodies were adsorbed against *E. coli* (Figure 6.3). Whilst signal generated for *E. coli,* decreases for BMM and Thermofisher polyclonal antibody (TRP), so does

the signals generated from the *Salmonella* serovars. Except *S.* Agama, the signals generated by BMM from *Salmonella* serovars are less than *E. coli*, the negative control, after adsorption. For TRP, only *S.* Agama and *S*. Typhimurium generate a signal greater than that of *E. coli* after adsorption. With Bio-rad polyclonal antibody (BRP), the signal generated by *E*. *coli* is greater after adsorption than before, (Figure 6.2 & 6.3) and the signals generated by *S.* Dublin and *S*. Mbandaka are less than that of *E. coli*. However, deviation between data sets was reduced.

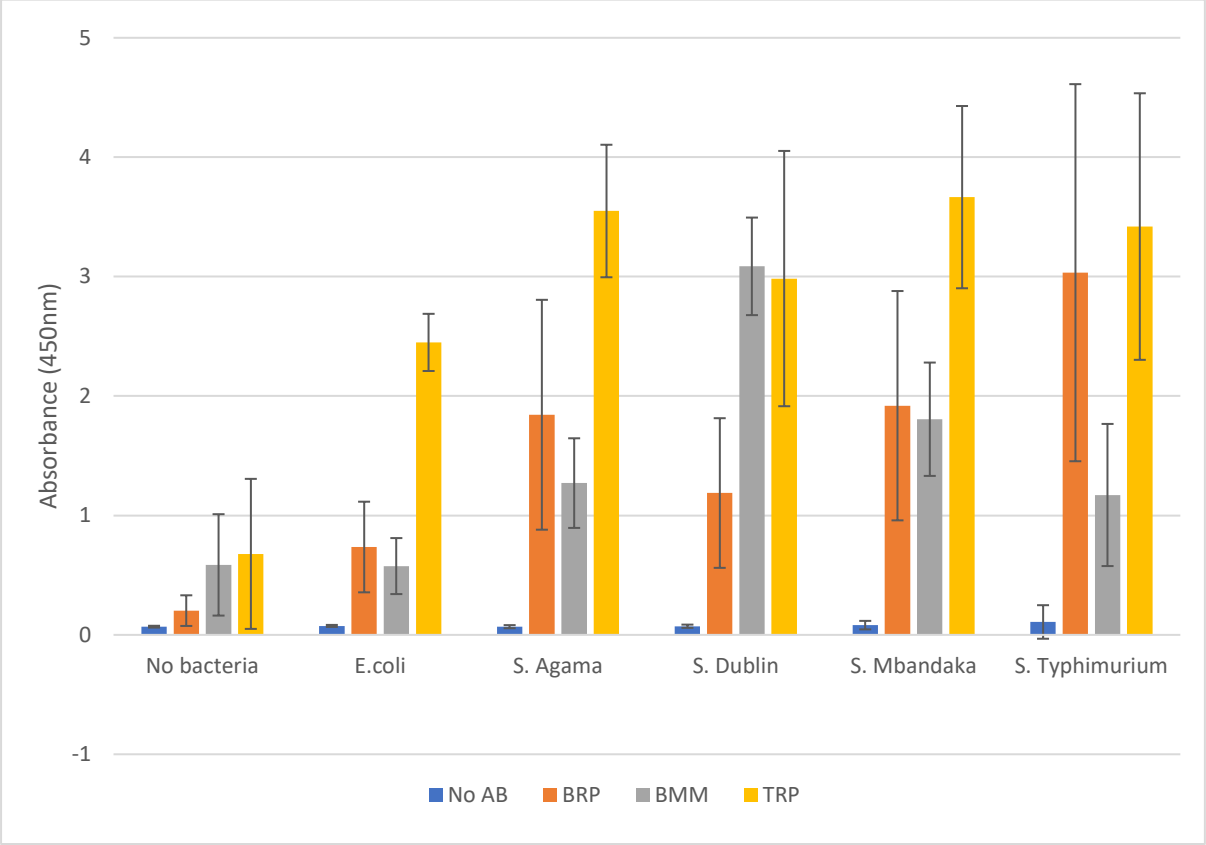

Figure 6.2: Detection of different *Salmonella* serovars by a panel of antibodies in a direct ELISA (n=5)

Key: No AB = 'no antibody' control, BRP = Bio-rad polyclonal antibody, BMM = Bio-rad monoclonal antibody, TRP = Thermofisher polyclonal antibody, error bars = standard deviation of data set

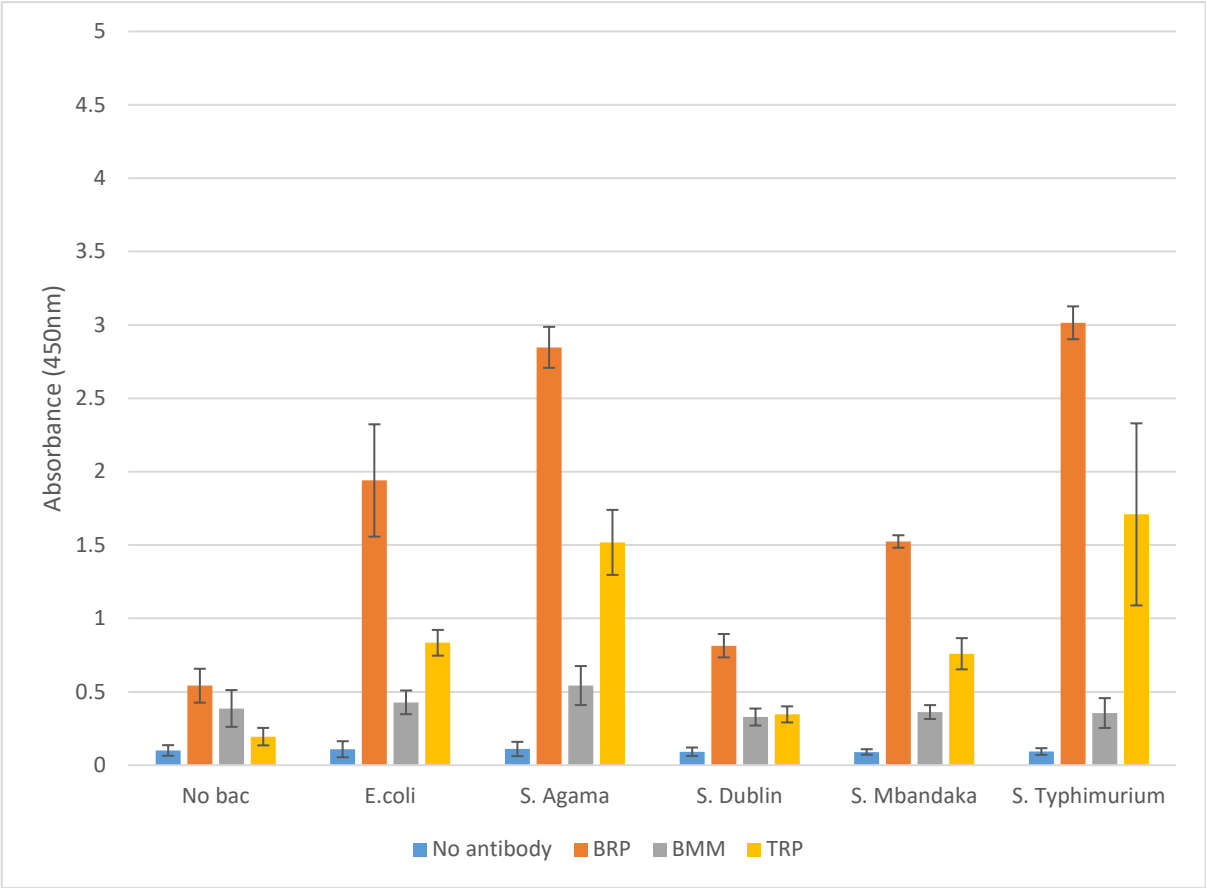

Figure 6.3: Direct ELISA, after adsorption of antibodies through *E. coli*, against different *Salmonella* serovars (n=8).

Key: No AB = no antibody control, BRP = Bio-rad polyclonal antibody, BMM = Bio-rad monoclonal antibody, TRP = thermofisher polyclonal antibody, error bars = standard deviation of data set

6.3.1.2 Optimisation of blocking concentration

To ensure non-specific binding sites were blocked effectively different concentrations of blocking buffer were tested. Blocking concentration affects both the absorbance at the endpoint of the assay, as well as the differentiation between result sets (Figure 6.4). It was clear that 0.1% skimmed milk in the blocking solution showed the best differentiation between *Salmonella* sp. and the negative controls, with the lowest deviation in results (Figure 6.4).

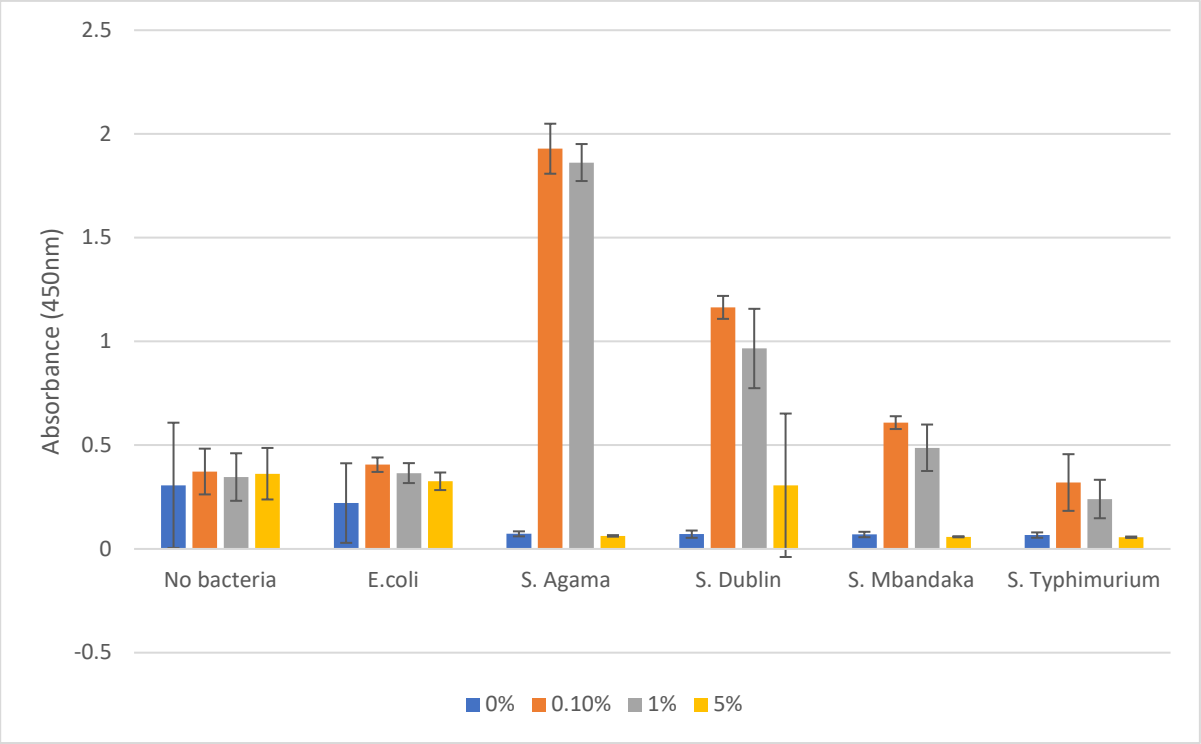

Figure 6.4: How differing concentration of milk within the blocking solution affect TRP detection of *Salmonella* strains in ELISA assays (n=8).

Key: 0% = no milk powder within blocking buffer, 0.10% = 0.1% w/v milk powder in blocking buffer, 1% = 1% w/v milk powder in blocking buffer, 5% = 5% w/v milk powder in blocking buffer, error bars = standard deviation of data set

6.3.1.3 Optimisation of Wash steps for ELISA 96 well plates

Due to the reduced signal generated by monoclonal antibody BMM when adsorbed against *E. coli*, another monoclonal antibody, A99H, was purchased and used as the monoclonal antibody in the following experiments. To reduce variability between ELISA plates, several wash steps were evaluated for this assay as described in section 2.8.3.3.1. From figure 6.5, it can be observed that ELISAs with washing steps using a multichannel pipette (MC) show a greater overall signal, as well as a more defined difference between the controls and *Salmonella* serovars, however there is greater deviation between repeats. ELISA assays with wash-bottle wash steps show greater repeatability and less deviation between repeats, however the overall signal generated is lower.

Examining the data for individual serovars, the pattern of deviation between repeats continues, with wash-bottle (WB) based washing steps producing less deviation between data sets (Appendix 4 & 5,

Figure 6.6). As bacterial number decreases absorbance also decreases for the majority of *S.* Dublin, *S*. Typhimurium and *S.* Mbandaka (Appendix 4 & Figure 6.6). However, for serovars *S.* Agama, *S.* Montevideo, and *S.* Newport, signal generation fluctuates as bacterial number decreases, sometimes without generating a signal greater than that seen for *E. coli* (Appendix 5). When comparing wash steps for *S.* Dublin and *S.* Typhimurium, overall WB wash step assays have a higher absorbance than that seen in MC wash step assays, which can also be observed with *S*. Mbandaka (Appendix 4). *S*. Mbandaka produced the highest absorbance response to A99H with good differentiation from *E. coli* going down to smaller bacterial numbers when compared to the other *Salmonella* serovars (Figure 6.6).

In Appendix 6, at $3.36x10^8$cfu/ml, A99H antibody produced a signal higher than that of the controls for all *Salmonella* serovars tested when ELISA plates were washed with multichannel pipettes. However, when plates were washed with a wash bottle, *S.* Newport and *S.* Montevideo both generated a signal lower than the no bacteria control, at $3.36x10^8$cfu/ml with A99H antibody (Appendix 7).

With wash-bottle plate washing, as the bacterial stock solution is diluted, the signal generated by conjugated A99H antibody decreases (Appendix 7). However, with multichannel washing, the signal produced does not follow this linear descent (Appendix 6).

A99H antibody produces the strongest signal in the presence of *S.* Mbandaka compared to other *Salmonella* serovars (Appendix 6A & 7A). For *S.* Mbandaka, *S.* Dublin, and *S.* Typhimurium, A99H antibody produces signal greater than that of the negative controls with signal not reducing below the controls until a 1/512 dilution of $3.36x10^8$cfu/ml (Appendix 6 & 7). The signal produced by A99H antibody in the presence of *S.* Agama, *S.* Montevideo, and *S.* Newport fluctuates above and below the controls, despite descending amounts of bacteria (Appendix 6B & 7B).

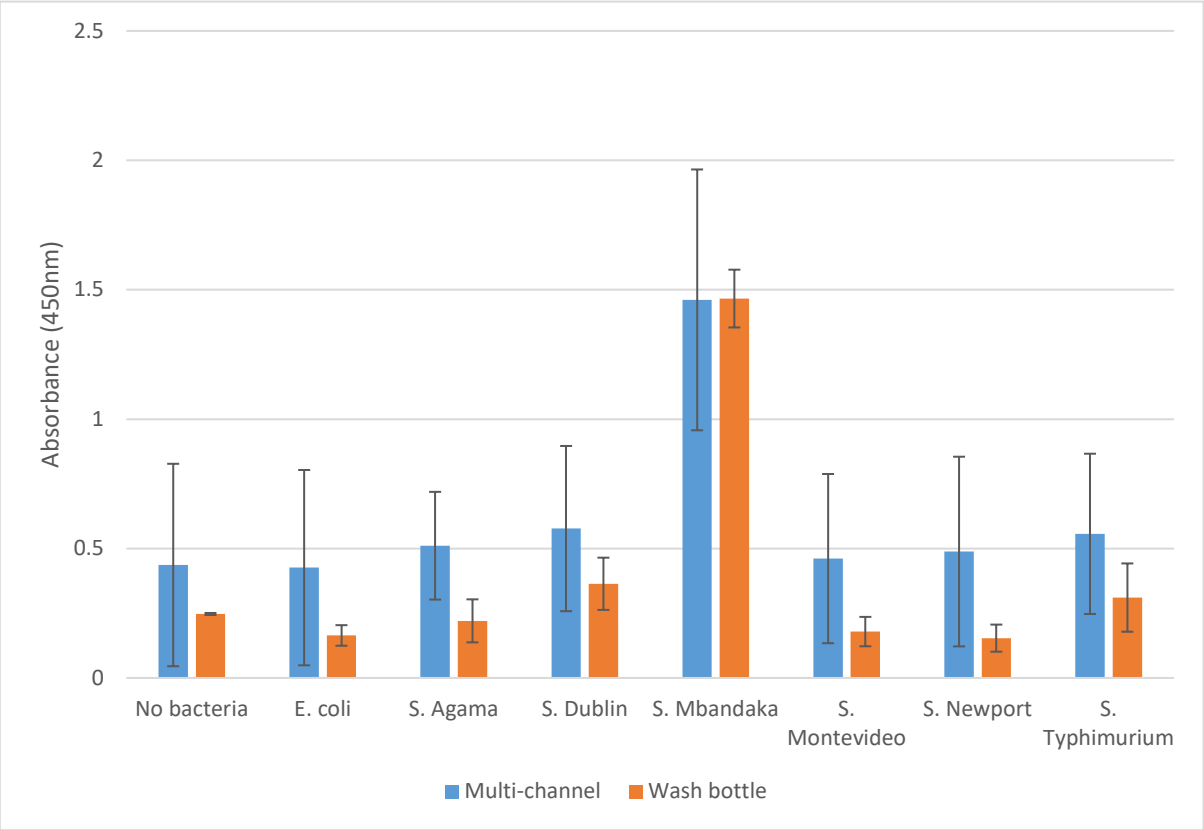

Figure 6.5: How different wash steps affect the efficacy of the direct ELISA assay of different *Salmonella* serovars at an average bacterial concentration of $3.36x10^8$cfu/ml. The antigen step incubated at 37°C and the antibody used was monoclonal A99H (n=3).

Key: Multi-channel = Multi-channel washes, Wash bottle = Wash-bottle washes

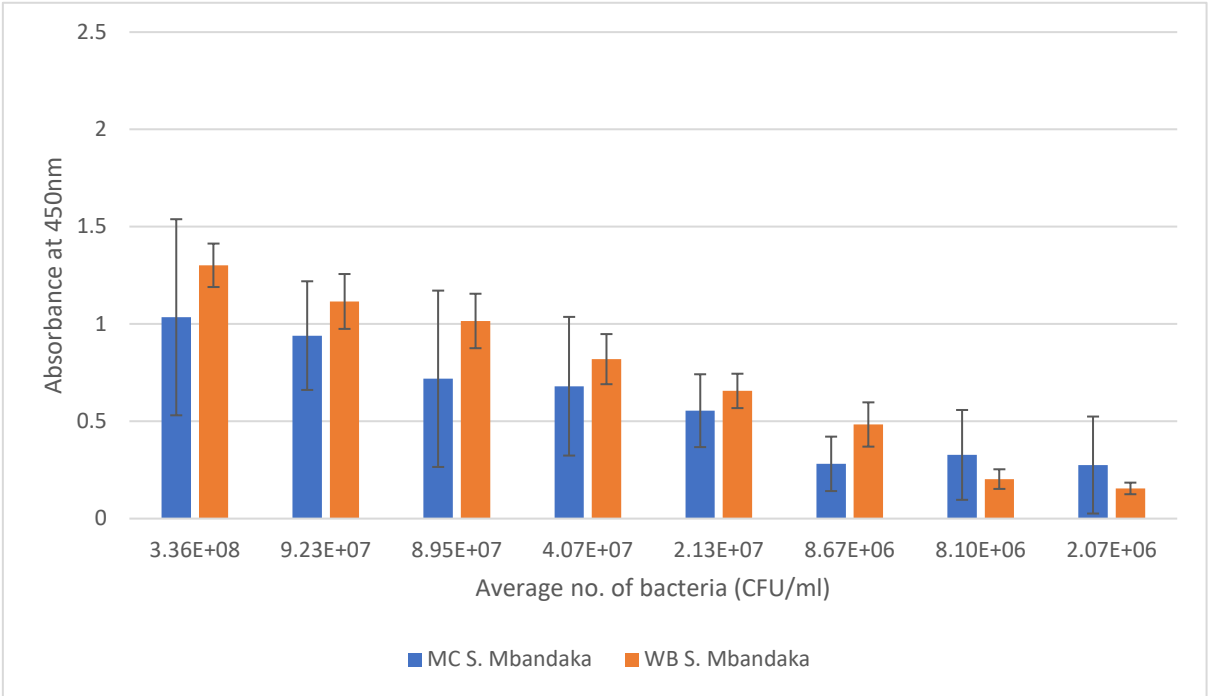

Figure 6.6: How different wash steps affect the efficacy of *S.* Mbandaka detection by monoclonal antibody A99H by direct ELISA over a range of different bacterial concentrations. Antigen step was incubated at 37°C (n=3).

Key: MC = Multichannel wash steps, WB = Wash bottle wash steps

6.3.1.4 Optimisation of ELISA incubation temperature

To optimise the direct ELISA assay, different incubation temperatures for the antigen incubation step were tested. In general, a larger absorbance was seen after antigen incubation at 37°C, however a reduced deviation between repeats was seen at 4°C incubation (See Figure 6.7, 6.8, & 6. 9). In appendix 8 this can mostly be seen with *S.* Dublin and *S.* Typhimurium, however at lower concentrations of bacteria, particularly for *S.* Typhimurium, greater absorbance is seen at 4°C. For *S.* Agama, *S.* Montevideo, and *S.* Newport there is not a linear decent in absorbance, despite a linear dilution of bacteria, and a large deviation between data sets is seen. At 4°C *S.* Newport rarely generates absorbance greater than that of the negative control (Appendix 9). In appendix 9, the absorbance generated by *S.* Agama, *S.* Montevideo, and *S.* Newport is reduced compared to other serovars, assays incubated at 4°C for these serovars generally produce absorbance lower than the negative control.

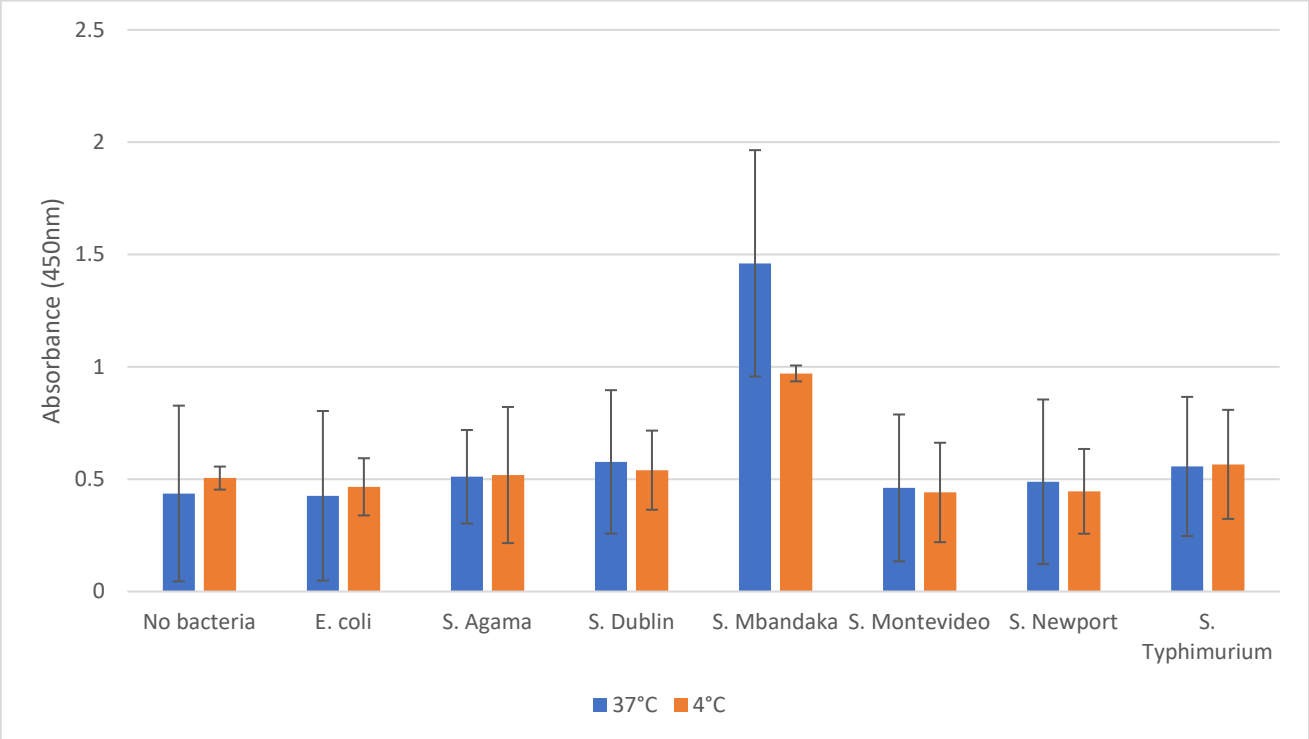

Figure 6.7: How differing the temperature of the antigen incubation step in direct ELISA protocol affects the efficacy of different *Salmonella* serovar detection at an average bacterial concentration of 3.36×10^8cfu/ml. Monoclonal antibody A99H and multichannel (MC) wash steps were used (n=3).

Key: 37°C = antigen incubated at 37°C, 4°C = antigen incubated at 4°C

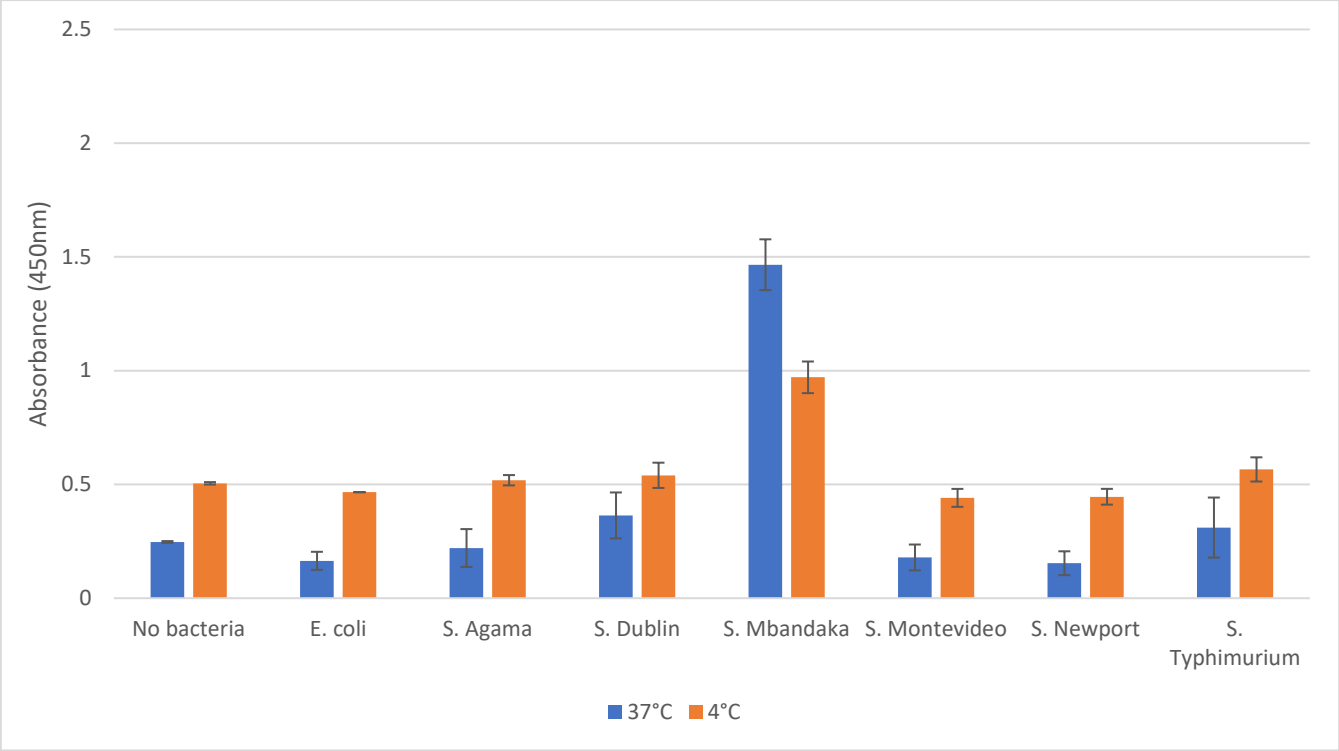

Figure 6.8: How differing the temperature of the antigen incubation step in direct ELISA protocol affects the efficacy of different *Salmonella* serovar detection at an average bacterial concentration of $3.36x10^8$cfu/ml. Monoclonal antibody A99H and wash bottle wash steps were used (n=3).

Key: 37C = antigen incubated at 37°C, 4C = antigen incubated at 4°C

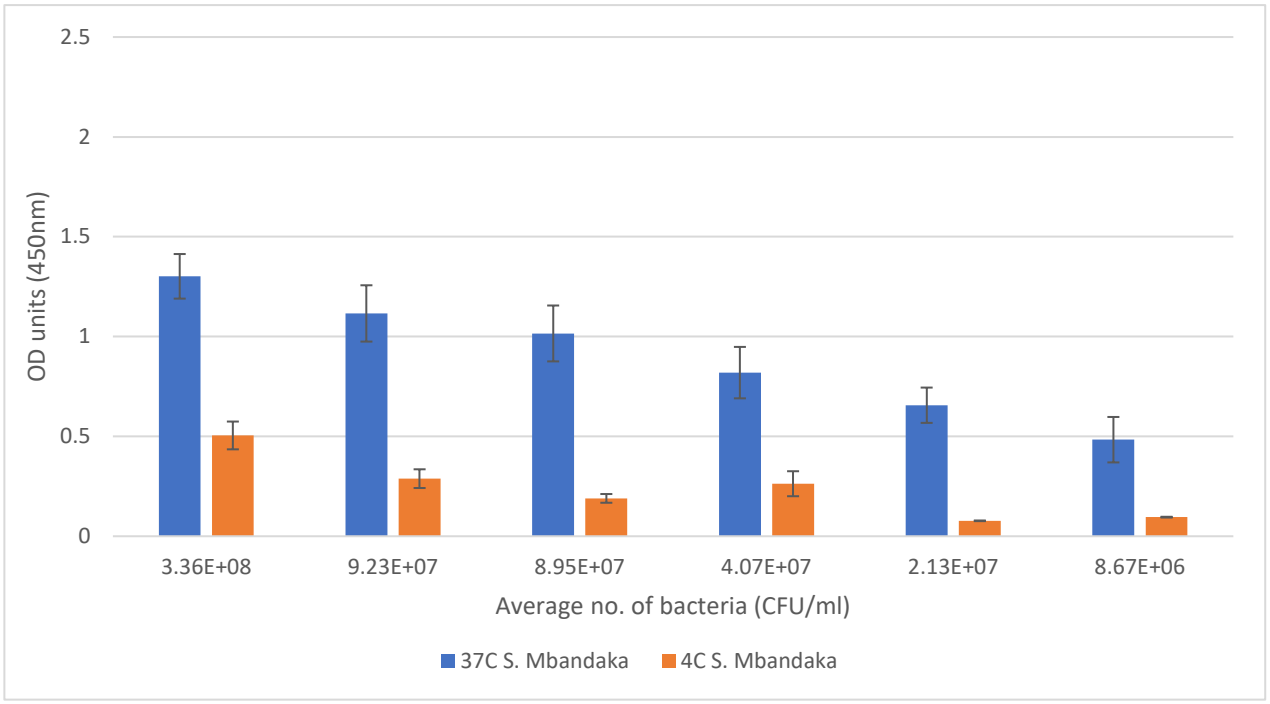

Figure 6.9: How temperature of the antigen incubation step in direct ELISA protocol affects the efficacy of *S.* Mbandaka detection by monoclonal antibody A99H by over a range of different bacterial concentrations. WB wash steps were used (n=3).

Key: 37C = antigen incubated at 37°C, 4C = antigen incubated at 4°C

6.3.2 Potentiometric immunoassays using Vantix Reader 1 (VR1)

Potentiometric immunoassays using the VR1 were completed as described in section 2.9.2. Direct Vantix assays were completed as described in section 2.9.2.2 and sandwich Vantix assays, adapted directly from the ELISA generated in this study, were completed as in section 2.9.2.3.

Direct assays were completed to determine whether clear definition between controls and test samples could be visualised using the VR1. Figure 6.10 shows a clear greater potentiometric signal for *S.* Dublin and *S.* Mbandaka compared to the control probes, using conjugated TRP antibody incubated with antigen for an hour. When antibody incubation times were reduced from 2 hours to 45 minutes a greater potentiometric signal was generated by test probes than by control probes (Figures 6.10, Appendix 10 & 11). At an antibody incubation time of 30 minutes, *S.* Mbandaka test probes produced signal less than the control probes (Figure 6.11).

The sandwich ELISA protocol, with an overall incubation time of 3.5 hours, was adapted to generate a Vantix immunoassay on the VR1. Before 14 seconds, *E. coli* control probe generates a potentiometric signal greater than that seen for the *S.* Mbandaka probe, however from 20 seconds to the end of the 90 second reading, *S.* Mbandaka generates a signal greater than that of both control probes (Figure 6.12). *S.* Dublin produces a greater potentiometric response than both control probes within the VR1 sandwich immunoassay (Figure 6.12).

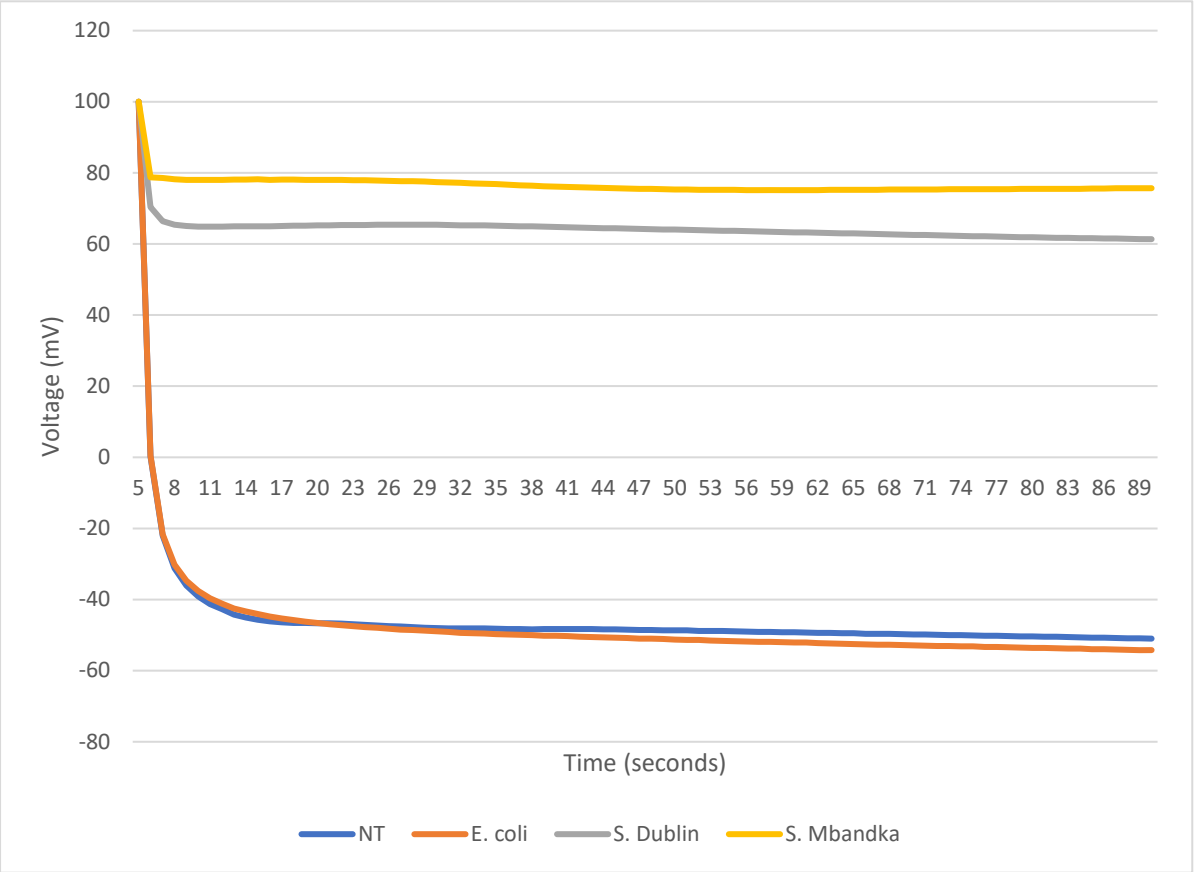

Figure 6.10: Average of direct immunoassays on the VR1, with conjugated polyclonal TRP antibody incubated for 1 hour. Bacteria was standardised to $8.95x10^{7}$cfu/ml (n=2).

Key: NT = Probe prepared with carbonate bicarbonate buffer in place of bacterial suspension

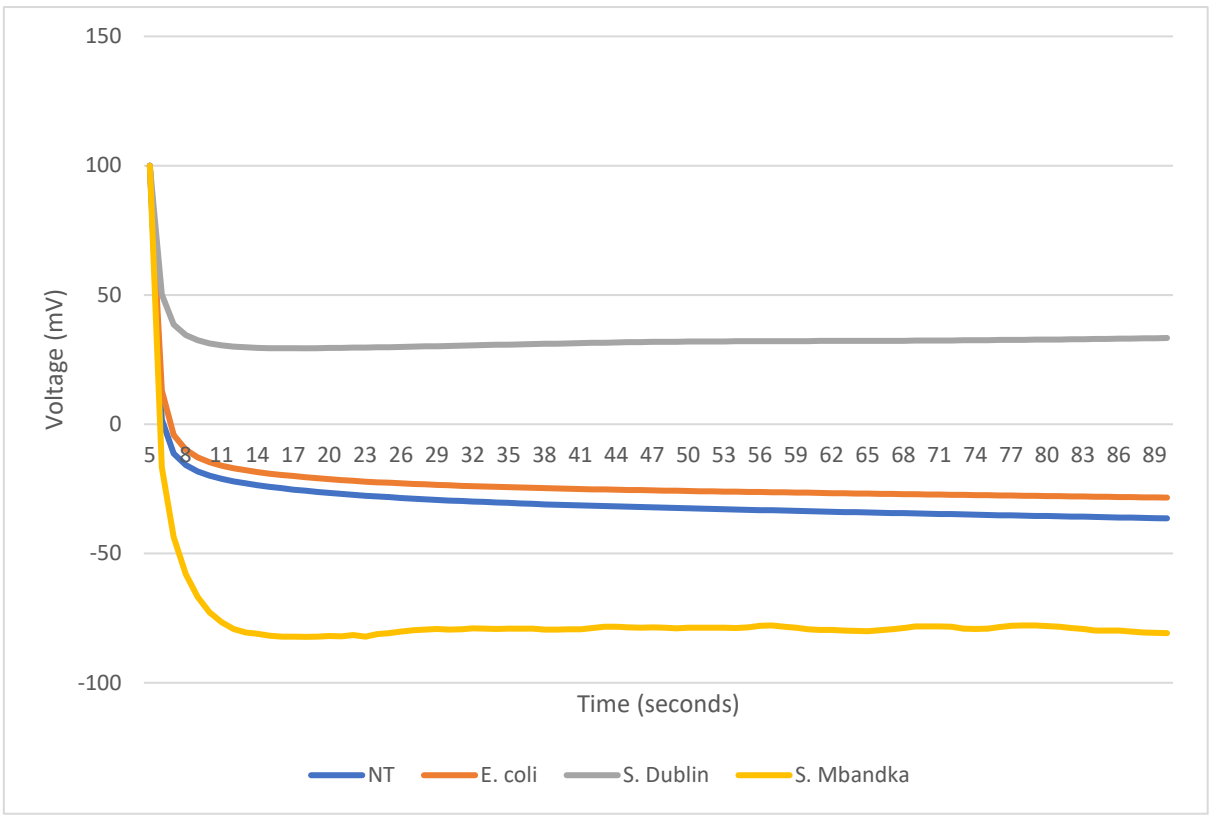

Figure 6.11: Average of direct immunoassays on the VR1, with conjugated polyclonal TRP antibody incubated for 30mins. Bacteria was standardised to $8.95x10^{7}$cfu/ml (n=2).

Key: NT = Probe prepared with carbonate bicarbonate buffer in place of bacterial suspension

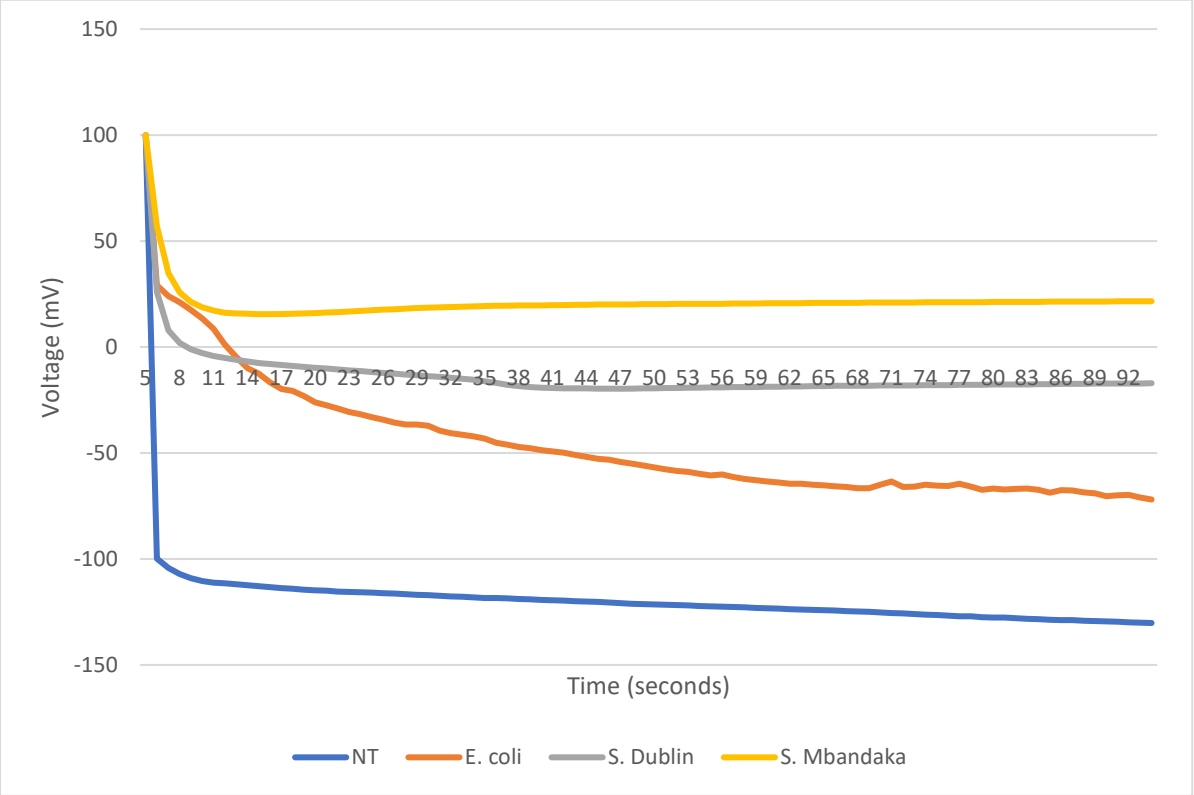

Figure 6.12: Average of sandwich assays run on the VR1, using polyclonal TRP antibody to capture and conjugated monoclonal BMM antibody to detect, with an overall incubation time of 2.5 hours. Bacteria was standardised to $8.95x10^7$cfu/ml (n=2).

Key: NT = Probe prepared with carbonate bicarbonate buffer in place of bacterial suspension

6.3.3 Potentiometric Sandwich immunoassays using the Vantix reader 2.0 (VR2)

Potentiometric immunoassays on the VR2 were completed as described in section 2.9.3. When probes are read in the VR2 reader, the first 10 seconds of signal fluctuate before stabilising, thus probe signals were interpreted after 10 seconds. The difference in voltage readings between controls and test probes was calculated by subtracting *E. coli* probe signal from *Salmonella* test probe. Throughout the following experiments TRP and A99H antibodies were used.

6.3.3.1 Optimising the concentration of the monoclonal A99H antibody

To determine the optimal operation concentration of A99H monoclonal antibody, two concentrations of the antibody were used in the Vantix sandwich assay. Overall a dilution of 1:100 for antibody A99H was shown to have the best potentiometric response produced by *Salmonella* sp. when compared to the controls (Tables 6.3 – 6.6, Figures 6.13 & 6.14, Appendices 12-17). *S.* Dublin produces an increased potentiometric response when compared to *E. coli* in all conditions (Tables 6.3 – 6.6), *S.* Mbandaka often produced a slightly higher response than *E. coli*, however on occasion its potentiometric response was lower than that of *E. coli* (Figure 6.13 and Appendix 16). Monoclonal A99H antibody concentration of 1:500 showed a reduced potentiometric response from *S.* Dublin and *S.* Mbandaka; however, the pattern of detection was similar to that seen with the 1:100 concentration.

From the VR1 testing, the sandwich assay had been reduced to 2.5hrs, this method and 2hr method were tested using two different concentration of A99H to see if monoclonal antibody concentration influenced assay time. With the 2hr method, a good differentiation between control and test samples was seen with 1:100 concentration of monoclonal antibody, suggesting that this concentration would allow for further optimisation of the assay with regards to incubation time (Table 6.3 and 6.5, Figure 6.13 & 6.14).

Table 6.3: Average difference in voltage readings of 2hr overall incubation immunoassays on the VR2 at two timepoints, with A99H monoclonal antibody at a 1:100 concentration (n=4)

A99H conc. = 1:100		**Voltage (mV)**		
Bacterial Average (CFU/ml)	Time (secs)	*E. coli*	*S.* Dublin	*S*. Mbandaka
8.95*10^7	30	411.2	445.9	404.7
	60	421.2	464.3	414.1
9.23*10^7	30	387.2	432.4	403.6
	60	394.1	450.0	413.4

Table 6.3A: Average coefficient of variation (%) of voltage readings of 2hr overall incubation immunoassays on the VR2 at two timepoints, with A99H monoclonal antibody at 1:100 concentration (n=4)

A99H conc. = 1:100		**Coefficient of variation (%)**		
Bacterial Average (CFU/ml)	Time (secs)	*E. coli*	*S.* Dublin	*S*. Mbandaka
8.95*10^7	30	4.7	5.2	4.7
	60	4.7	6.1	4.8
9.23*10^7	30	2.1	6.1	5.3
	60	1.7	6.0	5.8

Table 6.4: Average difference in voltage readings of 2hr overall incubation immunoassays on the VR2 at two timepoints, with A99H monoclonal antibody at a 1:500 concentration (n=4)

A99H conc. = 1:500		**Voltage (mV)**		
Bacterial Average (CFU/ml)	Time (secs)	*E. coli*	*S.* Dublin	*S*. Mbandaka
8.95*10^7	30	417.7	419.0	495.1
	60	429.6	431.7	509.1
9.23*10^7	30	406.6	422.2	421.1
	60	422.4	435.3	433.6

Table 6.4A: Average coefficient of variation (%) of 2hr overall incubation immunoassays on the VR2 at two timepoints, with A99H monoclonal antibody at a 1:500 concentration (n=4)

A99H conc. = 1:500		**Coefficient of variation (%)**		
Bacterial Average (CFU/ml)	Time (secs)	*E. coli*	*S.* Dublin	*S*. Mbandaka
8.95*10^7	30	5.0	5.8	29.9
	60	4.1	5.1	28.2
9.23*10^7	30	4.7	5.7	5.7
	60	5.3	5.0	5.0

Table 6.5: Average difference in voltage readings of 2.5hr overall incubation immunoassays on the VR2 at two timepoints, with A99H monoclonal antibody at a 1:00 concentration (n=4)

A99H conc. = 1:100		**Voltage (mV)**		
Bacterial Average (CFU/ml)	Time (secs)	*E. coli*	*S.* Dublin	*S*. Mbandaka
8.95*10^7	30	402.7	442.6	405.1
	60	409.4	461.4	413.0
9.23*10^7	30	406.4	428.4	405.3
	60	414.1	442.8	412.5

Table 6.5A: Average coefficient of variation (%) of 2.5hr overall incubation immunoassays on the VR2 at two timepoints, with A99H monoclonal antibody at a 1:100 concentration (n=4)

A99H conc. = 1:100		**Coefficient of variation (%)**		
Bacterial Average (CFU/ml)	Time (secs)	*E. coli*	*S.* Dublin	*S*. Mbandaka
8.95*10^7	30	2.6	4.6	2.6
	60	2.2	5.6	2.4
9.23*10^7	30	2.8	3.0	3.2
	60	3.1	2.9	2.9

Table 6.6: Average difference in voltage readings of 2.5hr overall incubation immunoassays on the VR2 at two timepoints, with A99H monoclonal antibody at a 1:500 concentration (n=4)

A99H conc. = 1:500		**Voltage (mV)**		
Bacterial Average (CFU/ml)	Time (secs)	*E. coli*	*S.* Dublin	*S*. Mbandaka
8.95*10^7	30	417.7	419.0	495.1
	60	429.6	431.7	509.1
9.23*10^7	30	406.6	422.2	421.1
	60	422.4	435.3	433.6

Table 6.6A: Average coefficient of variation (%) of 2.5hr overall incubation immunoassays on the VR2 at two timepoints, with A99H monoclonal antibody at a 1:500 concentration (n=4)

A99H conc. = 1:500		**Coefficient of variation (%)**		
Bacterial Average (CFU/ml)	Time (secs)	*E. coli*	*S.* Dublin	*S*. Mbandaka
8.95*10^7	30	3.5	3.5	3.6
	60	3.2	3.1	3.6
9.23*10^7	30	3.4	4.2	3.8
	60	3.1	4.2	3.7

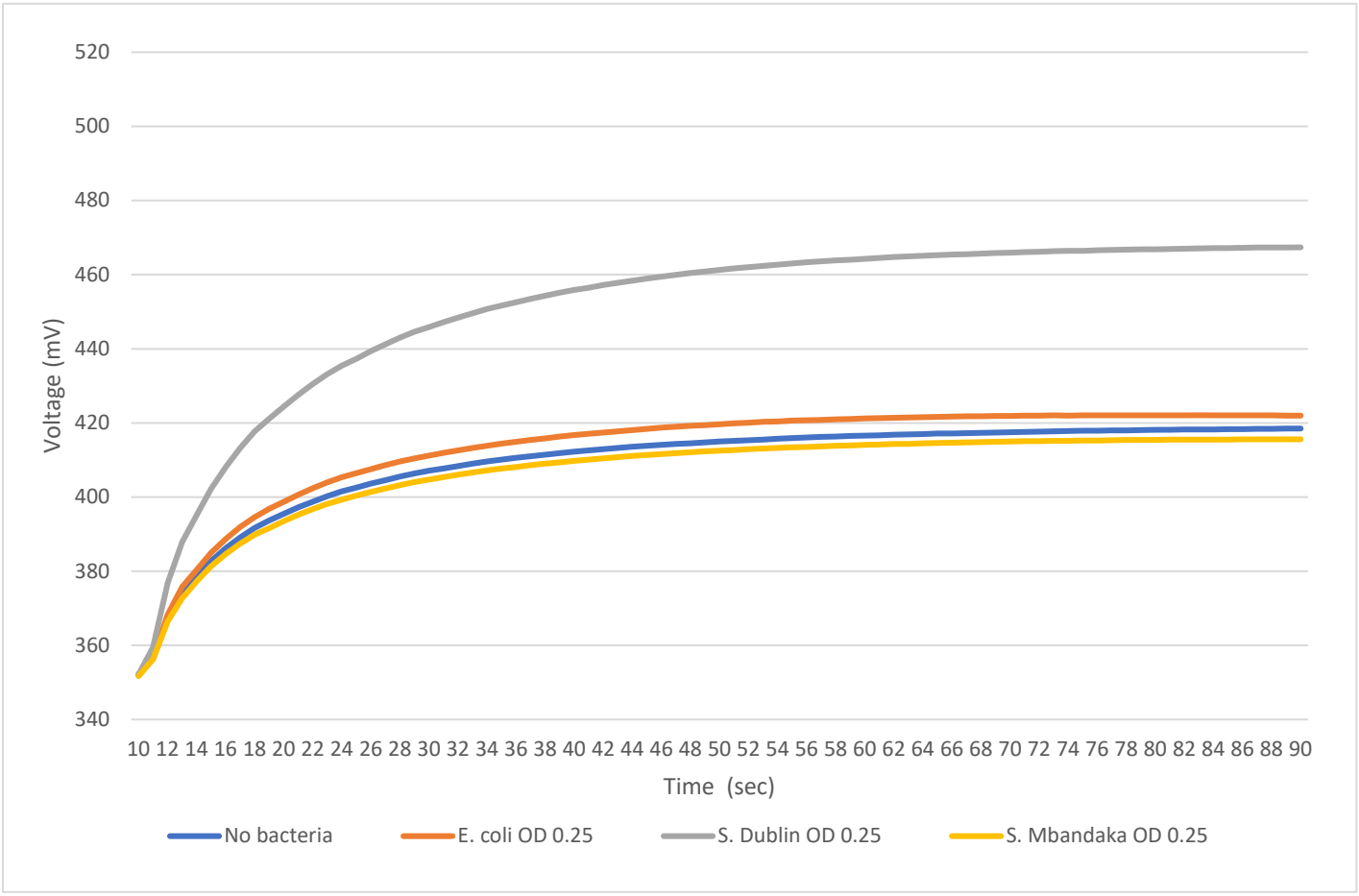

Figure 6.13: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 2hr overall incubation time. Bacteria were standardised at $8.95x10^7$cfu/ml. See Table 6.2A for the coefficient of variation at 30 & 60 seconds (n=4).

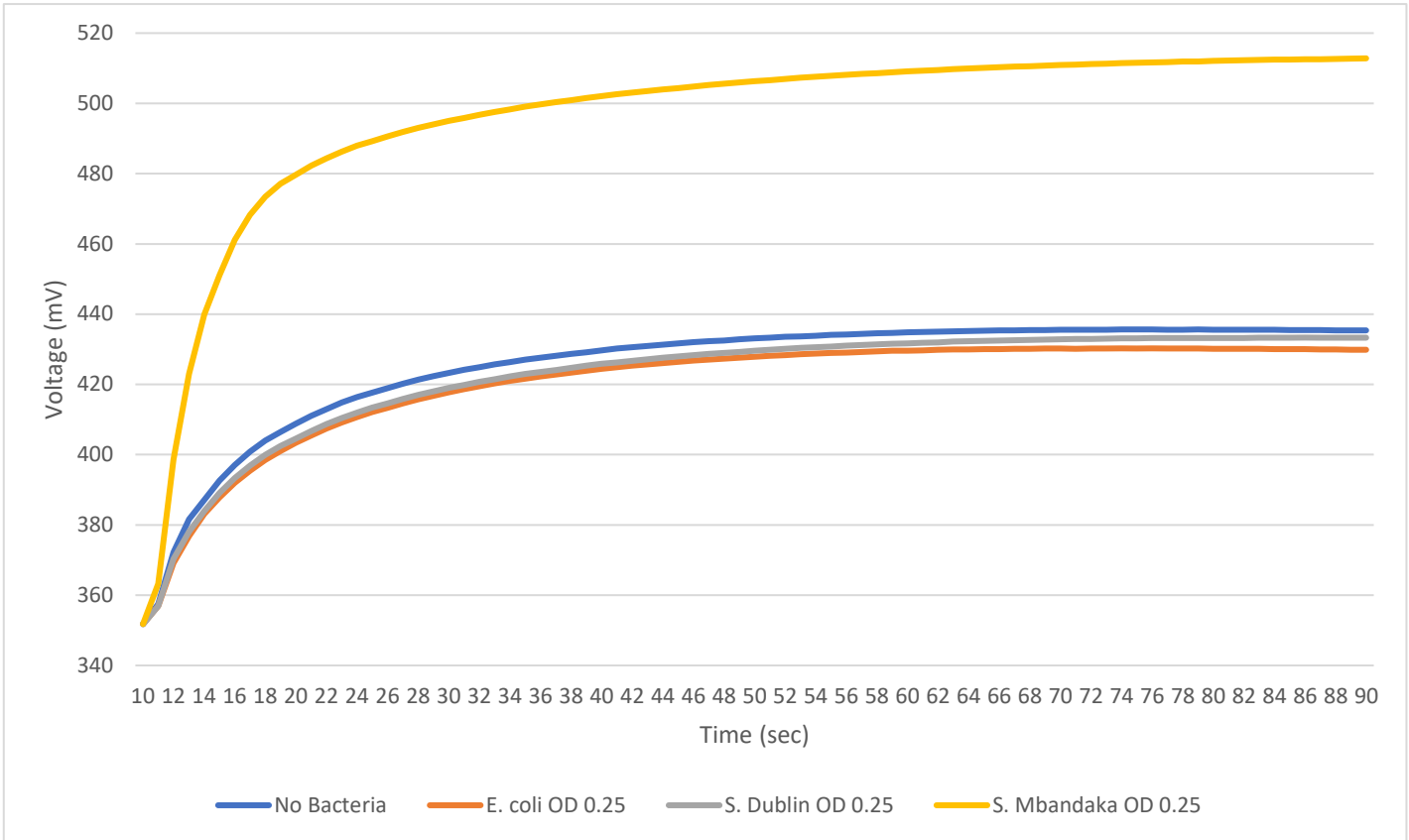

Figure 6.14: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:500 dilution, using 2hr overall incubation time. Bacteria were standardised at $8.95x10^7$cfu/ml. See Table 6.3A for the coefficient of variation at 30 & 60 seconds (n=4).

6.3.3.2 Optimising incubation step time for the Vantix sandwich assay

Further optimisation of the incubation times was undertaken to make the Vantix sandwich assay as rapid as possible without losing specificity. Antibody and blocking incubation steps were reduced to 15 mins and antigen incubation times of 60, 30, and 15 minutes were tested (overall incubation time; 1.75hrs, 1.25 hrs and 1hr respectively) as described in section 2.9.3.2.2.

At 1hr overall incubation, the differences between signals generated by control and test probes were not clear without taking the raw data and generating a graph to better differentiate between signals (Appendix 19 and 21). At 1hr incubation at $8.95x10^7$cfu/ml, *Salmonella* sp. test probes generate a greater signal than controls (Appendix 22) however at $9.23x10^7$cfu/ml, *Salmonella* sp. did not generate a potentiometric response greater than that of the controls (Appendix 23). Overall, at lower concentrations of bacteria, the Vantix Sandwich assay performed better and had greater differentiation between positives and negatives.

Whilst differentiation between signals was difficult, 40-minute overall incubation protocol was tested (Appendix 24 & 25). At $8.95x10^7$cfu/ml., *E. coli* generated a signal greater than both *Salmonella* test probes (Appendix 26), however at $9.23x10^7$cfu/ml both *Salmonella* test probes generated a signal greater than that seen for *E. coli* (Appendix 27).

Overall, a reduction of reagent incubation time has increased the differentiation between the potentiometric signals produced by the control and test samples. The best differentiation between controls and test sample was at 1.25hrs overall incubation, with both *S.* Dublin and *S.* Mbandaka producing a stronger potential than *E. coli* at both concentrations of bacteria at 30 seconds (Figure 6.15 and 6.16). At 30 seconds, *S.* Dublin produced a stronger response than *E. coli* at both 1.75 and 1.25hr incubations, whilst the response from *S.* Mbandaka was not as clear after 1.75hr incubation (Appendix 18 & 20).

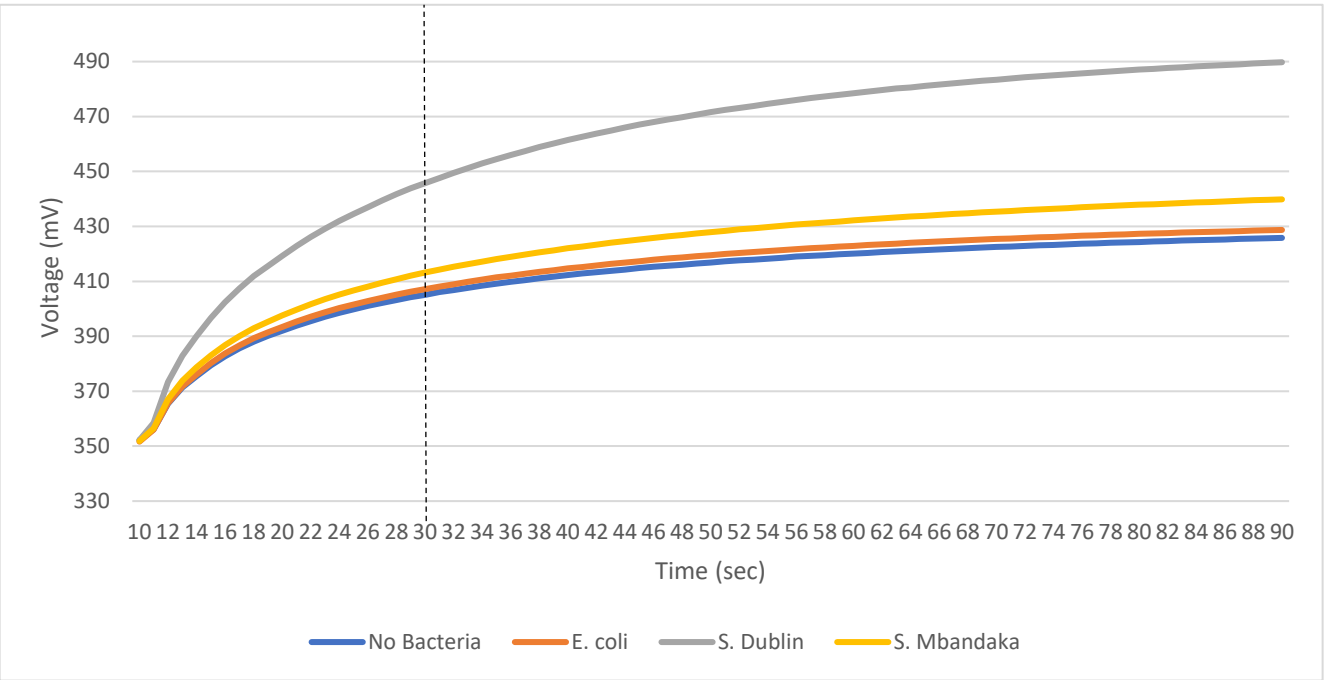

Figure 6.15: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 1.25hr overall incubation time. Bacteria was standardised at $9.23x10^7$cfu/ml (n=5).

Key: dashed black line = 30 second time point

Time (sec)	Coefficient of variation (%)			
	No bacteria	*E. coli*	*S.* Dublin	*S.* Mbandaka
30	6.08	6.80	10.36	7.11
60	6.31	7.16	12.26	7.24

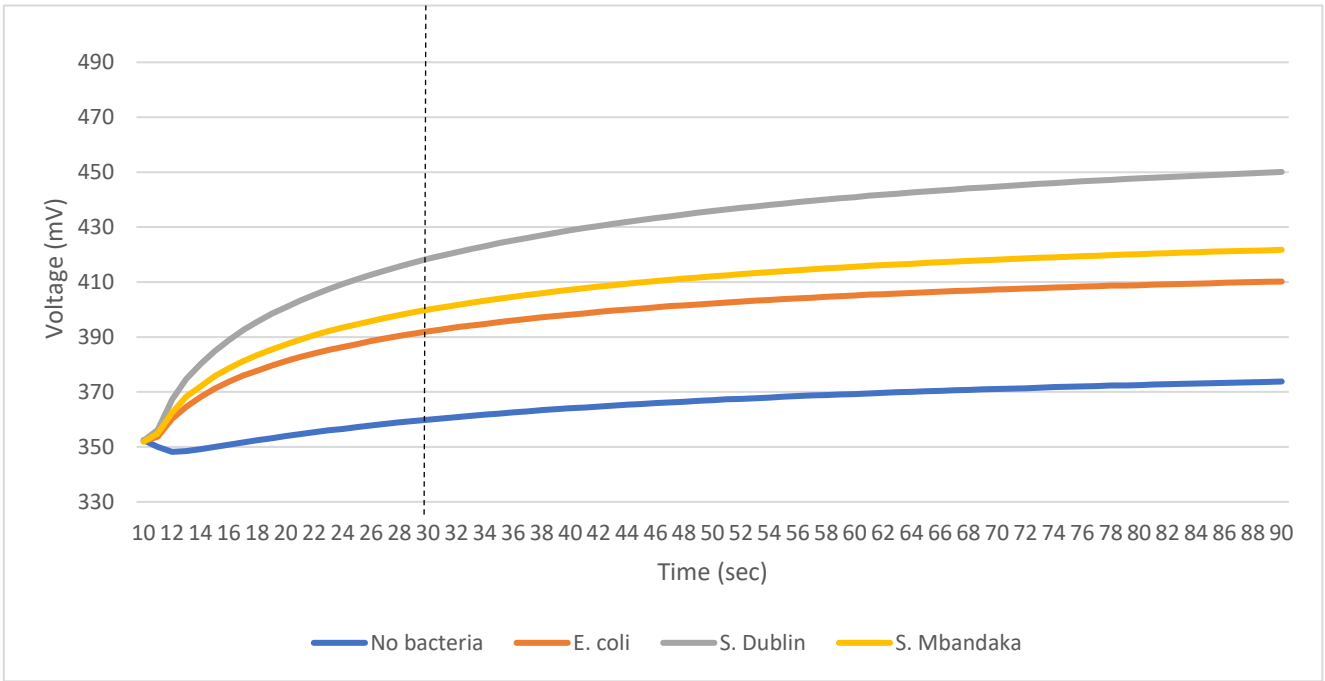

Figure 6.16: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 1.25hr overall incubation time. Bacteria was standardised at $8.95x10^7$cfu/ml (n=4).

Key: dashed black line = 30 second time point

Time (sec)	Coefficient of variation (%)			
	No bacteria	*E. coli*	*S.* Dublin	*S.* Mbandaka
30	14.66	5.73	4.97	4.45
60	14.80	6.09	4.85	4.62

6.3.3.3 *Specificity of the optimised potentiometric immunoassay to various Salmonella serovars using the VR2*

To test the specificity of the assay, a panel of *Salmonella* serovars was tested using the 1.25hr Sandwich Assay. Overall a clear differentiation between *Salmonella* serovars and the negative controls was seen at both 9.23x10^{7}cfu/ml & 8.95x10^{7}cfu/ml which is most apparent 30 seconds into probe reading (Figure 6.17 and 6.18). *S.* Typhimurium and *S.* Agama showed the strongest potentiometric response across both concentrations of bacteria (Figure 6.17 and 6.18). At 8.95x10^{7}cfu/ml *S.* Montevideo and *S.* Newport produced a similar voltage to that of *S.* Dublin over the 90 seconds of reading (Figure 6.17). At 9.23x10^{7}cfu/ml, *S.* Montevideo and *S.* Newport show a reduced potentiometric response, closer to that of *S.* Mbandaka and towards 70 seconds the plateau for both begins to decrease towards *E. coli* (Figure 6.18).

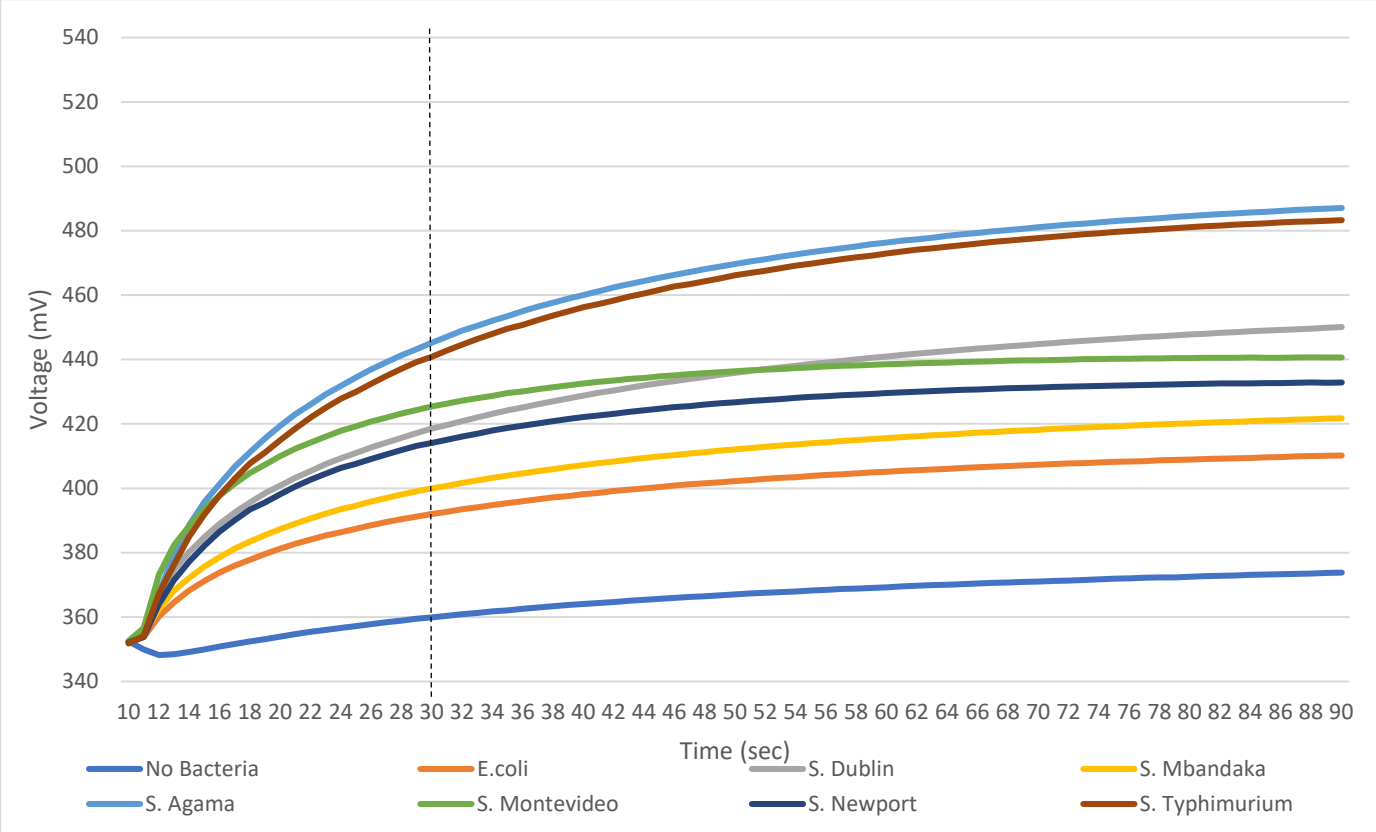

Figure 6.17: An average of Vantix Sandwich assays done on the VR2 testing different *Salmonella* serovars at 1.25hr overall incubation time, with bacteria standardised to 8.95x10^{7}cfu/ml (n=3).

Key: Black line = 30 second time point

Time (sec)	Coefficient of variation (%)							
	No bacteria	*E. coli*	*S.* Dublin	*S.* Mbandaka	*S.* Agama	*S.* Montevideo	*S.* Newport	*S.* Typhimurium
30	1.85	5.73	4.97	4.45	1.61	1.46	0.99	3.05
60	0.98	6.09	4.85	4.62	1.18	0.70	1.04	4.44

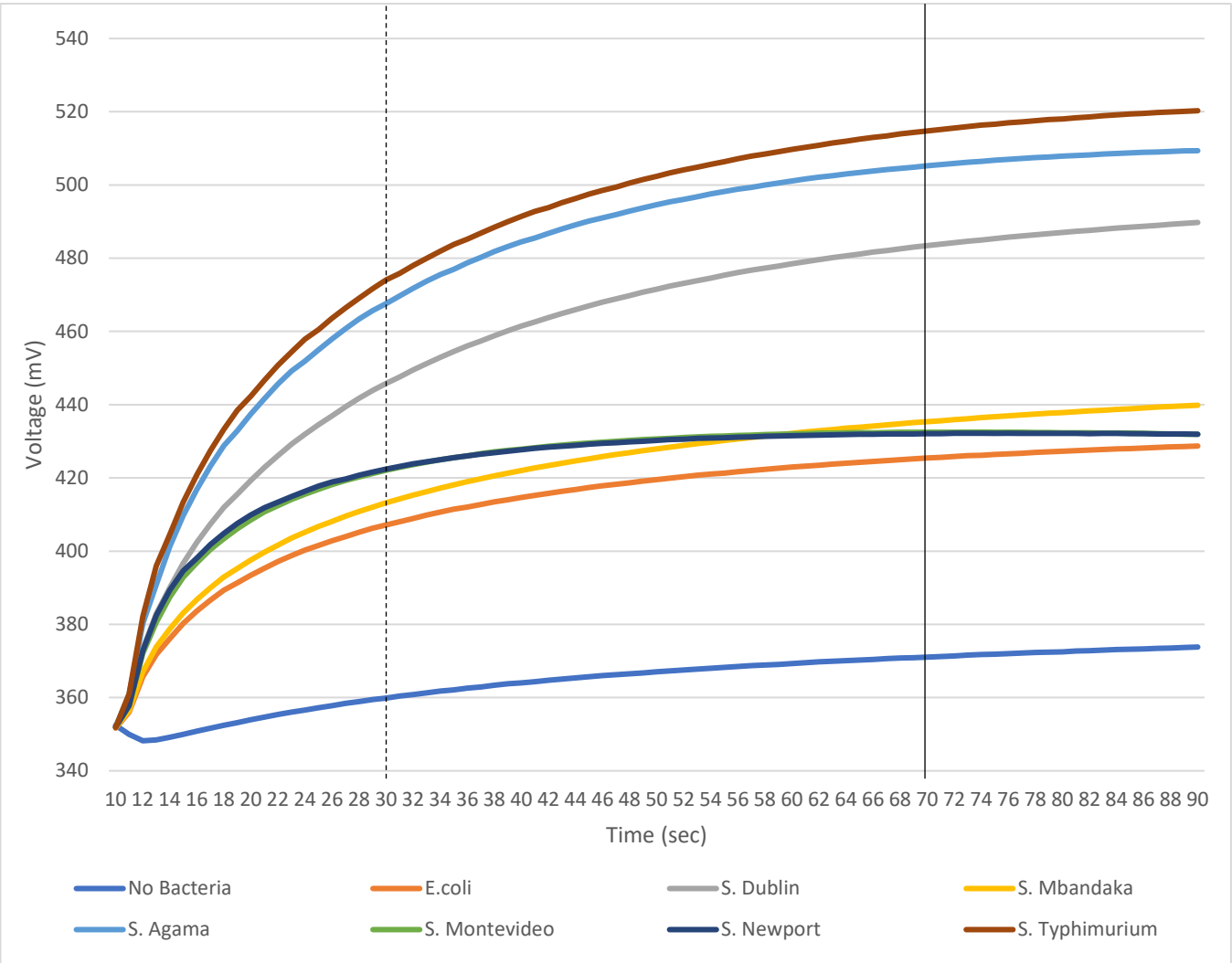

Figure 6.18: An average of Vantix Sandwich assays done on the VR2 testing different *Salmonella* serovars at 1.25hr overall incubation time, with bacteria standardised to 9.23x10^{7}cfu/ml (n=3).

Key: Dashed black line = 30 second time point, black line = 70 second time point

Time (sec)	**Coefficient of variation (%)**							
	No bacteria	*E. coli*	*S.* Dublin	*S.* Mbandaka	*S.* Agama	*S.* Montevideo	*S.* Newport	*S.* Typhimurium
30	1.85	6.80	10.36	7.11	2.46	0.86	0.90	0.47
60	0.98	7.16	12.26	7.24	3.87	0.42	0.70	2.18

6.3.4 Optimised potentiometric Sandwich immunoassay through calf scour using the VR2

To test whether calf scour could be used as a potential sample for this assay in a veterinary setting, calf scour was dosed with *Salmonella* sp. to challenge the assay as described in section 2.9.3.4. *Salmonella* negative scour was spiked with known quantities of *S.* Dublin for testing. Bacteria was standardised to an absorbance of 1 (~3.6x10^{8}cfu/ml), then diluted 1:2 to approximately

$2.13x10^7$cfu/ml. Within undiluted and diluted, 10µl of bacteria was added to 90µl faecal matter/solution, resulting in a 1/10 dilution of the bacterial concentration when compared to immunoassays not tested through scour. Due to this reduction in concentration, a reduction in signal is expected.

6.3.4.1 Salmonella detection through undiluted calf scour by sandwich immunoassays on the VR2

To test whether calf scour could be used directly as a potential sample for this assay in a veterinary setting, calf scour was dosed with *S.* Dublin at varying concentrations (approximately $3.36x10^7$cfu/ml, $9.23x10^6$cfu/ml, $8.95x10^6$cfu/ml, $4.07x10^6$cfu/ml, $2.13x10^6$cfu/ml) to challenge the assay. The following tests were done in duplicate and reading time was reduced to 60 secs. Overall, when read through calf scour the signal difference between *S.* Dublin & control probes was reduced, as expected due to the dilution of bacterial. However, at 30 seconds *S*. Dublin produced a stronger potentiometric response than the controls, except at the lowest concentration tested, $2.13x10^6$cfu/ml (Figure 6.19, Appendices 28-32).

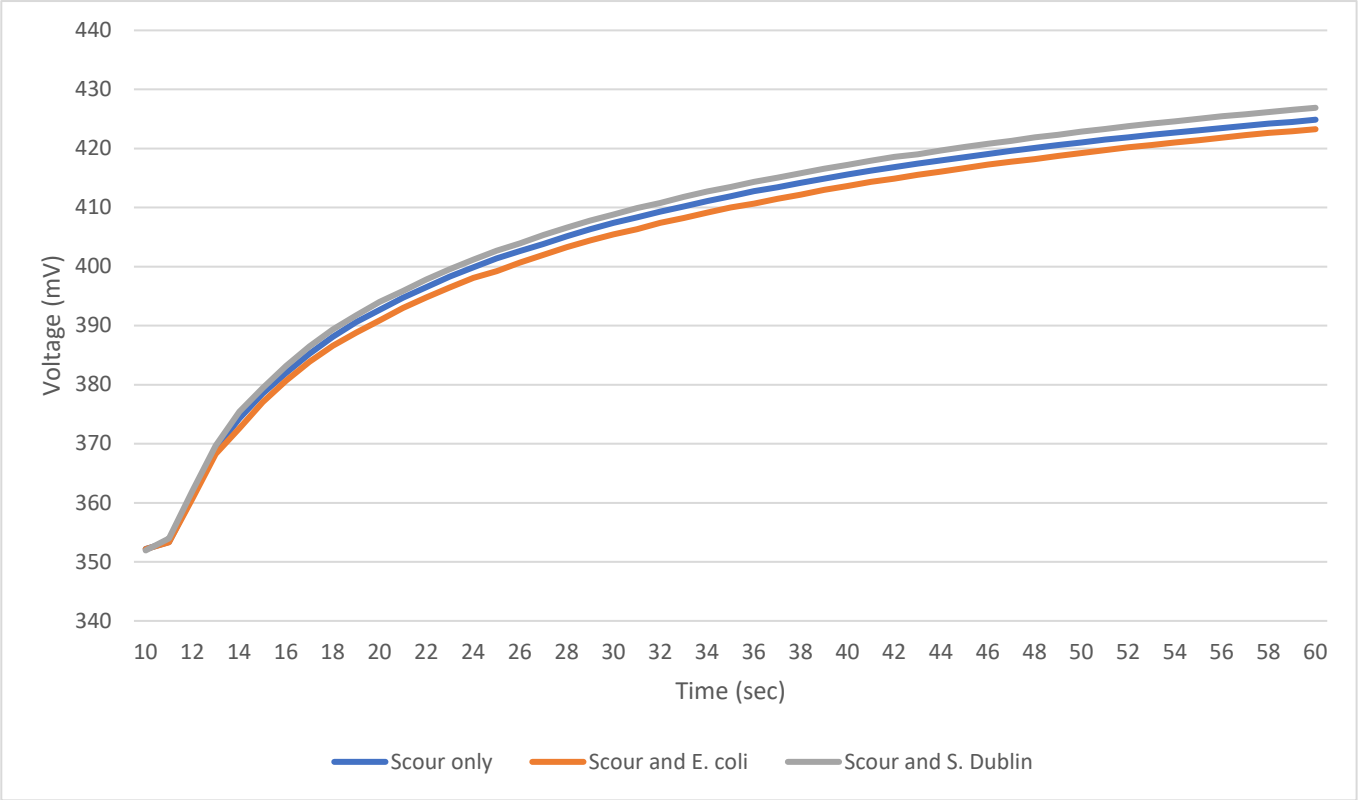

Figure 6.19: An average of the optimised Vantix sandwich assay completed through calf scour. Bacteria was diluted 1:10, to give approximately 8.95x10^{6}cfu/ml, in scour (n=3).

Time (sec)	Coefficient of variation (%)		
	No bacteria	*E. coli*	*S.* Dublin
30	2.99	3.18	4.02
60	3.63	4.16	5.17

6.3.4.2 Salmonella detection through diluted calf scour by sandwich immunoassays on the VR2

To determine whether dilution would reduce the antagonistic nature of scour, a 1:2 dilution of scour was undergone with carbonate bicarbonate buffer. A greater difference in signal between *S.* Dublin and *E. coli* through diluted scour was seen at 3.36x10^{7}cfu/ml - 8.95x10^{6}cfu/ml (Figure 6.20, Appendix 33 & 34), than seen through undiluted scour. At 4.07x10^{6}cfu/ml *S.* Dublin did not produce a higher potentiometric response than *E. coli* (Figure 20 and Appendix 35).

Interestingly, at 2.13x10^{6}cfu/ml, *S.* Dublin did not generate a potentiometric response higher than that of *E. coli* until 60 seconds, when previous positive potentiometric immunoassays could be read at 30 seconds. For the bacterial range of 8.67x10^{5} – 2.07x10^{5} cfu/ml, *S.* Dublin produced a potentiometric response lower than that of the negative controls (Appendix 37 – 39).

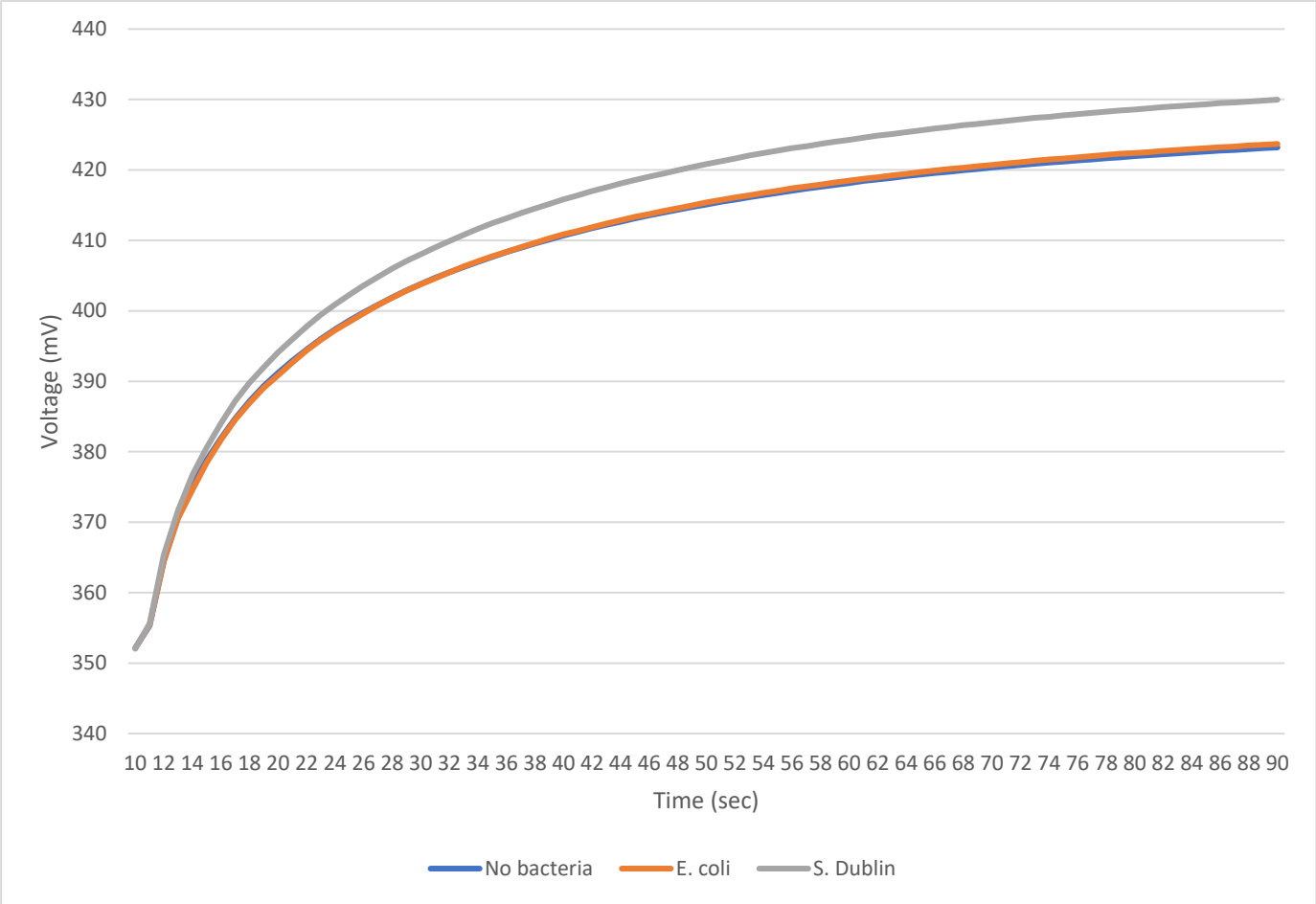

Figure 6.20: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted to approximately 9.23x10^{6}cfu/ml in scour (n=8).

Time (sec)	Coefficient of variation (%)		
	No bacteria	*E. coli*	*S.* Dublin
30	2.36	2.41	2.18
60	2.33	2.29	2.25

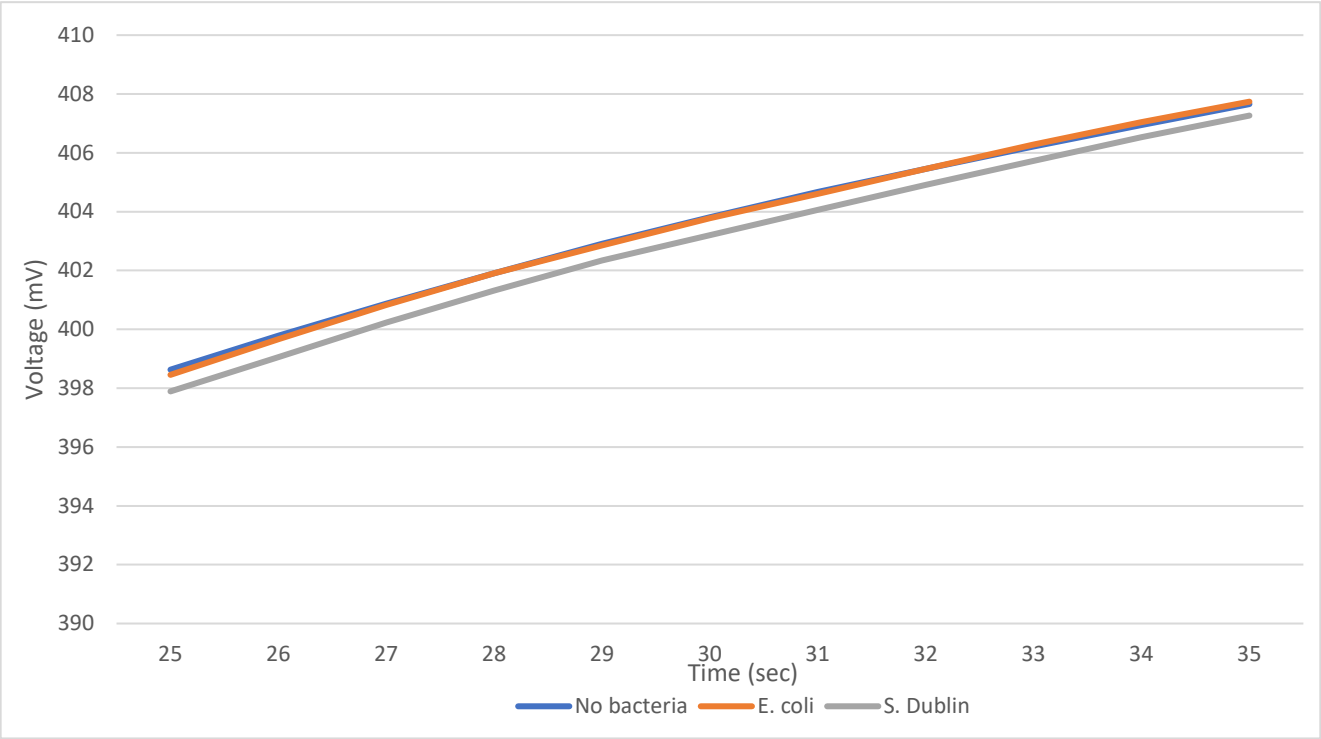

Figure 6.21: Closer look at appendix 35, the average of Vantix sandwich assays through a 1:2 dilution of calf scour, showing the 30 second time point. Bacteria was diluted to approximately $4.07x10^{6}$cfu/ml in scour (n=8).

Time (sec)	Coefficient of variation (%)		
	No bacteria	*E. coli*	*S.* Dublin
30	2.36	2.24	1.96
60	2.33	2.14	2.27

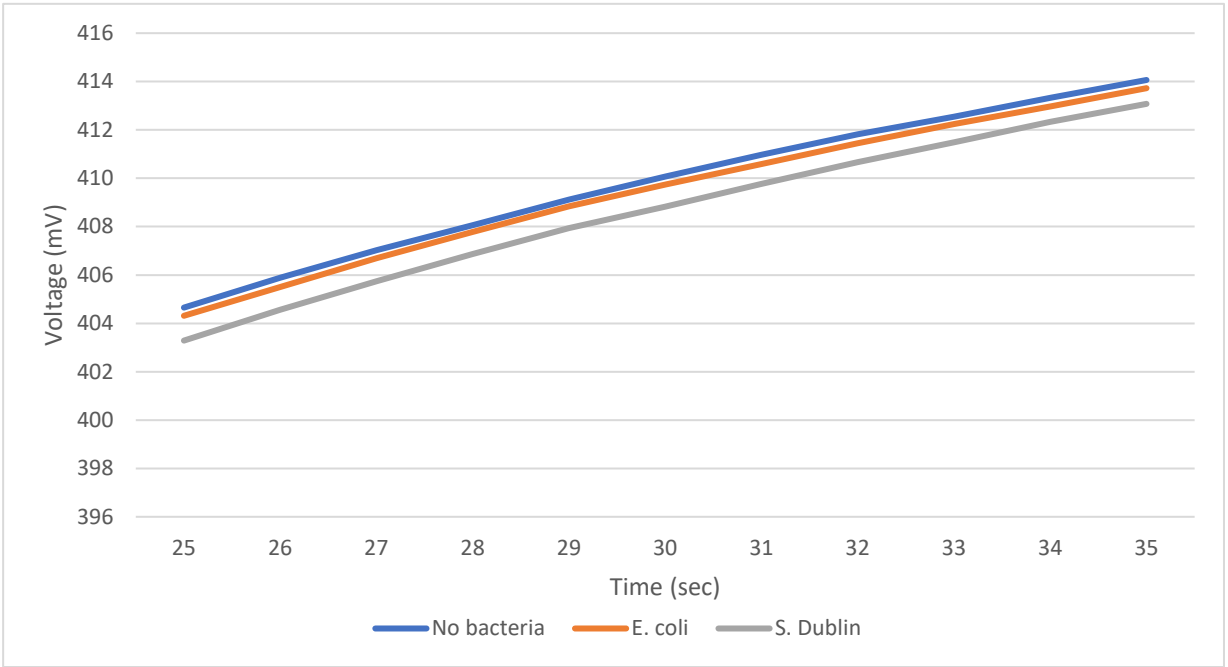

Figure 6.22: Closer look at appendix 36, average of Vantix sandwich assays through a 1:2 dilution of calf scour, showing the 30sec time point. Bacteria was diluted to approximately $2.13x10^{7}$cfu/ml in scour (n=12).

Time (sec)	Coefficient of variation (%)		
	No bacteria	*E. coli*	*S.* Dublin
30	3.02	3.45	2.65

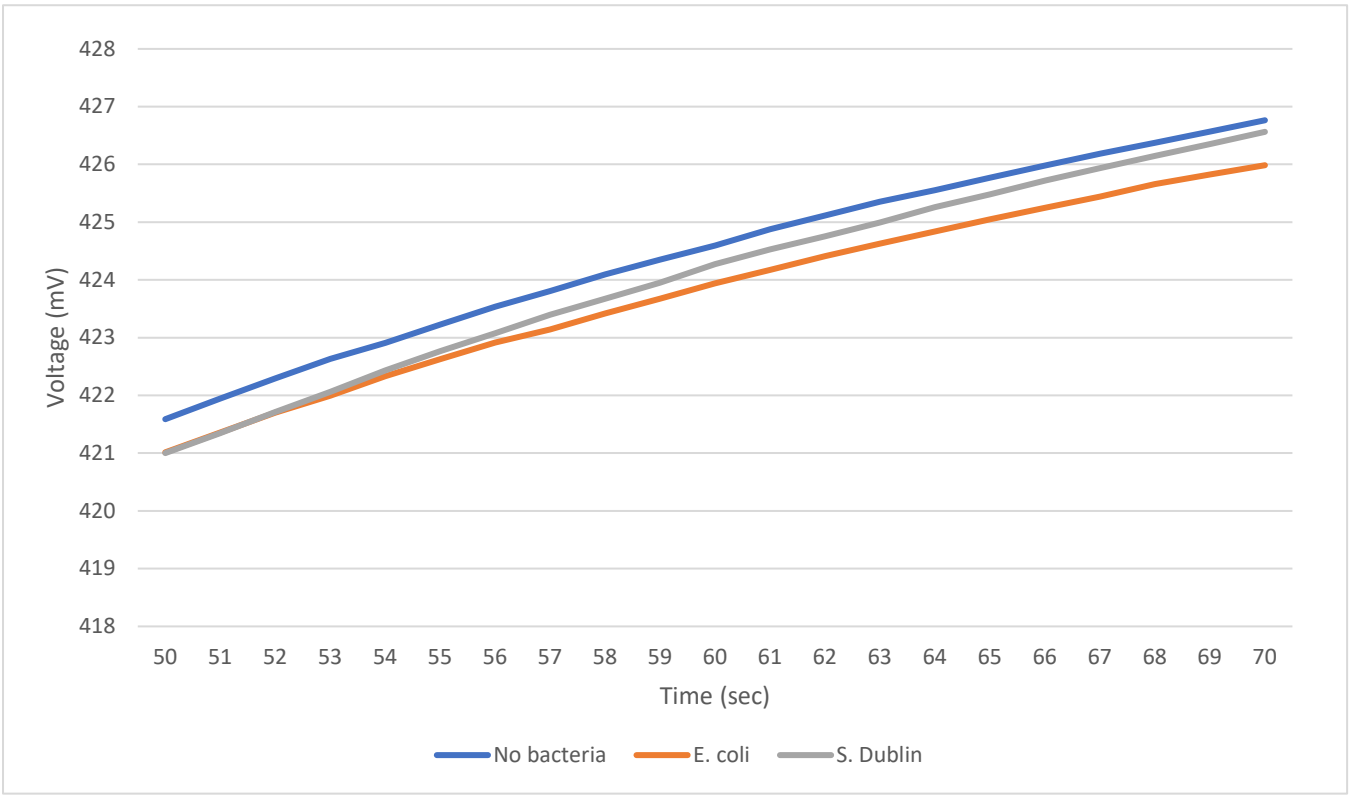

Figure 6.23: Closer look at appendix 36, average of Vantix sandwich assays through a 1:2 dilution of calf scour, showing the 60 second time point. Bacteria was diluted to approximately $4.07x10^6$cfu/ml in scour (n=8).

Time (sec)	Coefficient of variation (%)		
	No bacteria	*E. coli*	*S.* Dublin
60	2.33	2.14	2.27

6. 4 Immunoassay discussion

ELISA is an established technique that is considered the 'gold-standard' in immunoassays that can test large numbers of samples simultaneously and be convenient for operation with the potential for automation (Mobed *et al.,* 2019; Wu *et al*., 2014; Holford *et al*., 2012). Despite this, it can be a time-consuming protocol, including several working, incubation and washing steps, that Ewald *et al*. (2013) note doesn't allow for immediate countermeasures in case of an infection. Wu *et al*. (2014) note that conventional ELISA is limited by tedious sample pre-treatment and can suffer from poor sensitivity. Conventionally, ELISAs for the detection of *S*. Typhimurium requires pre-treatment with a broth-culture enrichment system, which is time-consuming (16-20hrs), or cell lysis, which can require expensive equipment or reagents (Wu *et al*., 2014; Cheung and Kam, 2012).

To circumvent these limitations, research into immunosensors has proved promising, maintaining the sensitivity and specificity of ELISAs but incorporating the rapid, ease of biosensors (Felix and Angnes, 2018; Bahadir and Sezginturk, 2015; Fei *et al.*, 2015; Holford *et al*., 2012).

Due to the benefits of immunosensors over conventional ELISAs, this study looks at generating an immunoassay using the Vantix system to target pan-*Salmonella* through calf scour. It has been shown that established ELISAs can be adapted to the Vantix platform, thus before generating a new Vantix immunoassay, an ELISA to detect pan-*Salmonella* was developed (Cork *et al*., 2012; Stead *et al*., 2011; Purvis *et al.*, 2003).

When generating rapid diagnostics, availability and ease of purchasing should be considered, to aim to reduce overall costs. Due to this, antibodies that were commercially readily available were chosen. Originally antibodies that targeted *S*. Dublin were sought, however due to the lack of these, antibodies that targeted core 'o' groups were found (Table 6.1), Bio-rad Mouse Monoclonal antibody (BMM), Bio-rad Rabbit Polyclonal antibody (BRP), & Thermofisher Rabbit Polyclonal antibody (TRP). To allow for integration of these antibodies into the Vantix system, a parent ELISA was generated to test the

efficacy of the antibodies before adapting the ELISA protocol to the Vantix system. When generating a protocol for the Vantix System, a parent ELISA assay is used, utilising the exact established reagents and principles, easily adapting them for use on the Vantix (Cork *et al*., 2012; Purvis *et al*., 2003).

Using ELISA, the antibodies' detection of *Salmonella* serovars was determined. All three antibodies (TRP, BRP, & BMM) detected pan-*Salmonella* at a higher absorbance that *E. coli* (Figure 6.2). When comparing the absorbance of each antibody to each *Salmonella* serovar, the response to *S*. Dublin was reduced compared to other strains for both polyclonal antibodies, however for Bio-rad monoclonal antibody (BMM) the response for *S.* Dublin was higher than for the other serovars (Figure 6.2).

Bio-rad reports that BMM recognises core antigen bearing O antigens, stating that serogroups O:2 (A), O:4 (B), O:7 (C1), O:8 (C2), O:9 (D), O: 3 (E1), & O:10 (E2) are recognised (Grimont and Weill, 2007). As *S.* Agama, *S.* Dublin, *S.* Mbandaka, & *S.* Typhimurium are all within the serogroups mentioned, a good absorbance for all the *Salmonella* serovars tested was expected. Whilst BMM did show more absorbance for salmonellae than for *E. coli*, there was a difference in absorbances across the *Salmonella* serovars. BMM showed a strong absorbance for *S.* Dublin, greater than that seen for TRP. The absorbance shown for *S.* Agama and *S.* Typhimurium with BMM was similar, which is what would be expected, as both are serogroup O:4 (B). The absorbance for *S.* Mbandaka was reduced compared to the absorbance for *S.* Dublin, with the absorbance of *S.* Agama and *S.* Typhimurium being greatly reduced compared to *S.* Dublin. This suggests that BMM was potentially raised against a *Salmonella* serovar from O:9 (D), due to the strong response to *S.* Dublin, compared to the other serovars, serogroups O:7 (C1) & O:4 (B).

TRP is reported to be raised to *S.* Enteritidis, serogroup O:9 (D), as well as *S.* Typhimurium & *Salmonella enterica* serovar Heidelburg, both serogroup O:4 (B) (Table 2.15 & 6.1; Grimont and Weill, 2007). TRP showed the most absorbance for *S.* Agama & *S.* Typhimurium, both are serogroup O:4 (B) so this is expected, however it also showed the most absorbance for *S.* Mbandaka, serogroup O:7 (C1), suggesting that this antibody potentially targets antigen/part of an antigen that is expressed

separately of serogroup (Table 6.1). Whilst TRP did not produce the highest absorbance for *S.* Dublin, compared to BMM, it did show a good absorbance, higher than that produced by *E. coli*, which would be expected as *S.* Dublin & *S.* Enteritidis both are serogroup O:9 (D).

BRP is reported to be polyvalent for *Salmonella* O & H antigens, however Bio-rad does not provide more detail as to what *Salmonella* serovars the antibodies were raised against. Compared to response of TRP in the presence of different *Salmonella* serovars, BRP produced an absorbance lower than that seen for TRP (Figure 6.2). However, BRP did produce an absorbance higher for all salmonellae tested compared to the absorbance in the presence of *E. coli*.

Bio-rad note that BRP is unabsorbed and may cross-react with related Enterobacteriaceae (Table 2.15). Additionally, it was noted that with TRP, that the signal generated by *E. coli* was high compared to the other antibodies and it was theorised that adsorption of the antibodies against *E. coli* could decrease this, increasing the specificity for *Salmonella* strains. However as seen in Figure 6.3, the overall detection of the *Salmonella* strains decreases, with *S.* Dublin and *S.* Mbandaka no longer generating a signal greater than *E. coli*, suggesting that the commercial antibodies are not as specific as potentially required for gastro-intestinal testing where *E. coli* and *Salmonella* sp. commonly co-exist. Also, BMM showed a greatly reduced signal once adsorbed against *E. coli*, no significant difference between *Salmonella* sp. and *E. coli* was seen, even though Bio-rad reports that this antibody should not cross-react with *E. coli*. Due to this monoclonal antibody A99H was purchased and subsequently used alongside the existing antibodies tested, as a more *Salmonella* specific monoclonal antibody was required (See Table 2.15). Conversely the signal generated by *E. coli* increased after adsorption for BRP. Despite the differentiation between repeats decreasing after adsorption, the low signal generation from *Salmonella* sp. after adsorption was considered detrimental to results and, considering current sample testing was single species, antibodies were not adsorbed against *E. coli* for immunoassays.

To reduce the variability between result sets, different steps of the ELISA protocol were optimised, starting with ensuring non-specific binding sites were effectively blocked by testing different concentrations of blocking buffer (Figure 6.4). Overall a concentration of 0.1% skimmed milk in the blocking buffer offered the best differentiation between *Salmonella* sp. and *E. coli*, as well as reduced deviation between repeats.

For further optimisation, wash steps were evaluated by using different washing techniques (Section 2.8.3.3.1). Washing with a wash bottle (WB) was advantageous as it was faster, required less precision and had less deviation between sets of results (Figure 6.6 & 6.7, Appendix 4 & 5). Wash-steps using a WB, resulted in a greater signal for *Salmonella* sp. when compared with *E. coli* with less deviation between results. This was the favoured washing method for improving reliability and sensitivity of ELISA results.

In appendix 6 and 7, despite a linear decrease in bacterial number, a linear decrease in signal generation was not observed, it appeared to fluctuate for serovars *S.* Agama, *S.* Montevideo, and *S.* Newport. The signals produced from *S.* Agama, *S.* Montevideo, and *S.* Newport are very low when compared to the other *Salmonella* serovars, sometimes not generating a signal greater than that of the negative control. This might suggest that antibody A99H does not efficiently recognise *S.* Agama, *S.* Montevideo, and *S.* Newport as it does other serovars, despite belonging to the O-groups the antibody is reported to target (Table 6.6). Conversely, *S*. Mbandaka shows the greatest signal, suggesting that A99H antibody has greatest affinity for this serovar, producing the highest signal with a large differentiation from the negative control, with a sensitivity greater than that observed with *S*. Dublin and *S.* Typhimurium (Appendix 6 and 7).

Incubation temperature for the antigen step of the ELISA was also tested, with incubation at 37°C and 4°C. Greater absorbance was seen at 37°C, however at 4°C there was less deviation between results. Overall, it was decided that incubation of the antigen at 37°C was more advantageous due to the level

of absorbance generated. Thus, ELISA assays were completed with incubation steps at 37°C, with WB washing steps in between.

From the ELISA assays, it was determined that TRP and A99H would be used as the capture and detection antibodies within the Vantix immunoassays. Additionally, the optimisations carried on from the ELISA were that immunoassays would be incubated at 37°C, with 0.1% casein within the blocking solution.

Once the protocol was optimised, the direct ELISA was adapted to a direct immunoassay on the Vantix research tool (VR1 direct immunoassay). The VR1 was used initially to optimise the immunoassay to the Vantix platform, by reducing overall incubation times. The direct ELISA assay took 6 hours overall, with 2-hour antibody incubation steps and this incubation time produced a good differential between negative and positive controls upon the VR1 (Appendix 10). This differentiation between positives and negatives was seen with an antibody incubation time to 45 minutes, however best differentiation was seen at 1 hour (appendix 11 and figure 6.10). After 30-minute antibody incubation, *S*. Mbandaka signal was less than that produced by *E. coli* (Figure 6.11). Whilst antibody incubation steps had been decreased, the ELISA sandwich assay was adapted to the VR1, with the original antibody incubation steps, showing clear difference in potentiometric signal between *E. coli* and *Salmonella* probes after 20 seconds (Figure 6.12). Overall, sandwich assays were chosen going forward. Due to the antagonistic nature of Scour, as well as the complexity of molecules present in faeces, the sandwich assay is preferable as it is reported to be more sensitive than direct assays, useful when using a complex sample (Konstantinou, 2017).

Whilst reducing the overall incubation times of the direct VR1 immunoassays, the aliquots of reagent on to the black test probes were reduced from 5µl to 3µl. The surface tension of reagent within the slight well of the probe held better, ensuring aliquot volumes were more uniform, as aliquots were not flooding over the well edge, no reduction in reactivity was observed.

When using the VR1, several platform limitations were discovered. The software that provides the output of the VR1 immunoassays was difficult to navigate and use, graphs would be generated but no accompanying raw data was produced with no visual explanation. Troubleshooting determined that excel needed to be installed to allow for raw data production. Additionally, probes for the VR1 come pre-printed in a long strip, and individual probes need to be cut and prepared separately. Due to their light-weight nature individual probes can be disturbed easily, by something as simple as a colleague walking by, which can lead to loss of probes, reagents, as well as load order. Additionally, individual probes are inserted separately into the VR1 reader, upon assembly of the platform, the probes are prone to coming loose, as there is nothing attaching them to the reader. These issues are in line with what Cork *et al*. (2012) reported and they suggest that the VR1 could be improved to enable the system for routine laboratory application and increase user-friendly nature. By optimising the reader and the accompanying software, as well as developing combs of probes that are compatible with 96-well plates to prevent the need for handling of individual probes, Cork *et al.* (2012) suggest the relative simplicity and speed of the Vantix platform would be useful for point-of-care testing. These improvements have been addressed by the development of the Vantix Research Tool 2 (VR2).

The difficulties faced with the probes in the VR1 are resolved with the VR2. The VR2 probes come in combs of 12 and are spaced to fit within a 96 well plate, removing the need to handle individual probes. Comb clips have been included within the new platform to enable easy attachment to the VR2 reader. Additionally, the VR2 software is more user-friendly and enables viewing of raw data within the software, with the option to export, making the entire platform easier and more intuitive to the user.

To continue adapting the ELISA assay to the Vantix platform, the 2.5-hour overall incubation time VR1 direct immunoassay was tested upon the VR2, alongside a 2hrs overall incubation time immunoassay, reducing antigen incubation time from 60 to 30 mins. Whilst previous antibodies had been used at a concentration of a 1: 500, Thermofisher recommended antibody concentration of 1:20 – 1:200 for

monoclonal A99H antibody when used in ELISA assays. Differing concentrations of the monoclonal antibody A99H were used, 1:100 and 1:500, to see if an increase in concentration increased the detection of *S.* Dublin. Overall a 1:100 concentration of A99H generated a greater potentiometric response overall than a concentration of 1:500, therefore a 1:100 concentration was used for the following immunoassays (Table 6.3 -6.6, Figures 6.13 & 6.14).

Incubation times for the VR2 immunoassays were reduced further, with antibody and blocking incubation steps were reduced to 15mins, with antigen incubation times of 60-, 30-, and 15-min incubation times (overall incubation time; 1.75hrs, 1.25hrs and 1hr respectively). Until 1hr overall incubation, a reduction in reagent incubation times resulted overall in an increased differentiation between the potentiometric signals produced by control and test samples, with the best differentiation seen at 1.25hrs incubation for both *S.* Dublin and *S.* Mbandaka. The VR2 sandwich immunoassay was run at 1.25hrs for the following assays. Overall, the VR2 sandwich immunoassay showed a greater differentiation between test and control samples at lower concentrations of bacteria.

Operational adjustments were made whilst adapting the immunoassays from the VR1 to the VR2. It was noted when adding TMB substrate, a large quantity was needed within a trough with no physical barrier between probes. As the VR2 comb of probes fit within a 96 well plate, with an individual well for each probe set, TMB substrate was added to a 96-well plate when testing. An aliquot of 300µl per well was needed to cover both probes, reducing the overall amount of TMB substrate used. Additionally, with a physical barrier between probes, less differentiation between data set was observed, increasing reliability of the results, and suggesting that probes could be affected by the potentiometric reactions on neighbouring probes. Whilst some connectivity issues had been improved by the redesign of the VR2 probe combs, some issues were still observed. When attaching combs, clips needed to be changed each time to ensure a good connection was establish, producing a lot of plastic waste between combs. Additionally, great care needed to be taken when attaching combs, as any

damage to the connecter pad of the probe resulted in a loss of connection and therefore result. However, once a technique was established, connector damage was easy to avoid. The addition of the 'play' button to the VR2 reader allowed for connection testing before adding substrate, enabling the user to establish that all probes were connected before running.

From the ELISAs and the differences in signal generation for *S.* Dublin and *S.* Mbandaka from the VR immunoassays, it had already been determined that more specific antibodies, targeting pan-*Salmonella* would be needed than those commercially available. However, to determine what change in specificity the VR2 sandwich assay had in comparison to the direct ELISA assay developed within this study, a panel of *Salmonella* serovar was tested via sandwich immunoassays on the VR2. Overall a clear differentiation between the *Salmonella* serovars tested and the negative controls were seen at both concentrations of bacteria tested (Figure 6.17 & 6.18).

Graphs were read at 30 seconds to determine differentiation between controls and test samples, which is an improvement upon the direct ELISA developed within this study, where *S.* Agama, *S.* Montevideo and *S.* Newport rarely generated a response greater than that of the negative control. Within the VR2 sandwich assay *S.* Typhimurium and *S.* Agama produced the strongest potentiometric response (Figure 6.17 & 6.18). The capture antibody, TRP, is reported to be raised against *S.* Typhimurium, which shares a serogroup with *S.* Agama, O:4 (B), suggesting that TRP has a high affinity for this serogroup (Table 6.6). The high potentiometric signal from *S.* Agama within the VR2 sandwich immunoassay is interesting as within the direct ELISA the affinity between A99H and *S.* Agama appeared to be poor. At a bacterial concentration of 9.23×10^7 (Figure 6.18), *S*. Montevideo, *S.* Newport and *S.* Mbandaka show lower response than that of the other serovars, however at a bacterial concentration of 8.95×10^7 (Figure 6.17), *S*. Montevideo and *S.* Newport show an increased response closer to that of *S.* Dublin. *S.* Mbandaka and *S.* Montevideo are both within O:7 (C_1), with 6, 7, and 14 as somatic antigens. *S.* Newport is in a separate O group but shares somatic antigen 6, which could be a potential antibody target to improve responses to these serovars (table 6.1).

When looking at the bacterial concentration of $9.23x10^7$cfu/ml at around 70 secs *S.* Montevideo and *S.* Newport plateau and decrease in potentiometric signal, decreasing towards the negative control (Figure 6.18). This could potentially be to substrate depletion and suggests that it is useful to read the graphs towards the beginning of signal generation, rather than at the end.

Bahadir and Sezginturk (2015) note that academically developed biosensors should be applied on real samples to determine their practical use. Cork *et al.* (2012) note that it needs to be demonstrated the Vantix technology works with samples that realistically be taken in on-site testing situations, without the need for laboratory-based equipment, such as a centrifuge. To test whether the VR2 sandwich assay could be used to detect *Salmonella* sp. through calf scour in a veterinary setting, calf scour was spiked with *Salmonella* sp. at varying cfu/ml dosages. A 1:10 dilution of bacteria in a range of concentrations within calf scour was used and showed vastly muted potentiometric response, likely due to the reduction of bacterial concentration as well as potential assay inhibitors found in faecal matter. However, until a bacterial dilution of 1/10 of $2.13x10^6$ cfu/ml, *S.* Dublin produced a potentiometric response higher than that of the negative control, showing that the potentiometric immunoassay developed on the VR2 can detect *S.* Dublin through calf scour, an antagonistic sample.

To increase the potentiometric signal seen within a positive result, a 1:2 dilution of the calf scour was tested against the same bacterial concentrations, resulting in a stronger potentiometric response, suggesting that within calf scour there are assay inhibitors. By diluting calf scour, a stronger differentiation between positive and negative samples was seen and sensitivity was increased. *S.* Dublin produced a potentiometric response stronger than that of the negative controls at $2.13x10^6$ cfu/ml - $8.67x10^5$ cfu/ml, a potentiometric response stronger than the negative controls was not seen in the tested bacterial concentration lower than this.

Whilst test probe response curves were often easily distinguishable above control response curves without manipulation of raw data, at lower concentrations of bacteria differences in potentiometric signal were harder to read. With rapid detection method, instant understanding of the generated

results is required to keep the speed of the diagnostic. Development of a control probe, by creating a standard curve of potentiometric signals at different bacteria concentrations, would be, useful to allow for quick differentiation and to allow for targeted treatment of *Salmonella* infection on farm.

The potential for a pan-*Salmonella* immunoassay that can detect through faecal matter has been demonstrated within this study. The sensitivity of an immunosensor is reported to be strongly connected to the affinity of the antibody to antigen (Fei *et al*., 2015; Haji-Hashemi *et al*., 2019; Purvis *et al*., 2003). Cork *et al.* (2012) propose that BoHV-1 assay performance could be improved by use of more specific antigen, as a relatively crude whole viral antigen was used. The same can be said with the pan-*Salmonella* VR2 sandwich assay developed within this study, more specific antibodies would allow for a greater potentiometric response as well as greater detection for multiple serovars. Marega *et al*. (2020) present a streamlined, rational approach to developing mixtures of antibodies, maximising the antigen-antibody affinity to specificity profiles of 54 *Salmonella* strands. Future work would investigate raising anti-*Salmonella* antibodies with the ability to detect multiple *Salmonella* serovars, to improve the potentiometric response of the Vantix sandwich assays to *S.* Mbandaka, *S.* Newport and *S.* Montevideo without losing the response of other *Salmonella* sp.

Cork *et al.* (2012) noted that Vantix is pragmatic and practical system, which is commercially available at a reasonable cost and can be used without specialist knowledge of biosensors: the biosensors produced for Vantix use an established screen-printing technology, offering the prospect of cheap mass production. Cork *et al.* (2012) highlight an important advantage of the Vantix platform, in that it produced an electronic numerical result, suitable for transmission via mobile networks, should interpretation or decision making regarding downstream disease control and treatment need to be made utilising portable equipment. This study shows the relative ease with which a parent ELISA can be generated and adapted to the Vantix system. The adaptability of the Vantix system could be an important advantage to the acceptance and adoption of the technology: the ability to use exact established ELISA reagents and assay principles is advantageous (Cork *et al*., 2012; Purvis *et al*., 2003).

Given the widespread use of antibody detection in diagnostic testing, The Vantix platform could be meet the requirements of a wide variety of diagnostic tests (Cork *et al.*, 2012). Overall, the VR2 system is a vast improvement on that of the VR1 and allows for the adaption of established ELISA assays to a fast and easy to use potentiometric biosensor assay that can be used on-site, be that at a veterinary clinic or on farm, without the need of extensive sample preparation.

8. References

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7. Discussion

Bovine salmonellosis represents a major economical and welfare challenge in the cattle industry worldwide (Wallis *et al.*, 1995). Infection with *S.* Dublin can lead to unacceptable levels of morbidity and mortality, with calves often dying within 48 hours of infection (Nielsen, 2013). Nielsen *et al.*, (2004) noted several of the economic losses caused by *Salmonella;* such as the death of calves and young animals, abortions and reproductive disorders. Furthermore, a loss of product is seen due to poor growth of infected animals adding to economic loss caused by salmonellosis in cattle (Jadidi *et al.*, 2012). Infection often results in additional labour costs and additional veterinary expenses. Control measures such as isolation, treatment and culling often need to be implemented, having a negative economic effect on the farmer (Mateus *et al.*, 2008).

Foodborne pathogens throughout the food chain are a major concern for the industry and public health (Malorny *et al.*, 2004). Silva *et al.* (2011) note that the presence of salmonellae in food, make it unsuitable for human consumption. Salmonellosis symptoms can range from gastrointestinal infections with inflammation, diarrhoea and vomiting, to typhoid fever, a life-threatening systemic infection (Hensel, 2004). To ensure food safety and to safeguard public health, the rapid, reliable, and specific detection of pathogenic bacteria is crucial (Silva *et al.*, 2011; Santos *et al*., 2014; Wang *et al*., 2018).

In microbiological diagnosis, stool culture is the standard method for diagnosing *Salmonella* gastroenteritis (Falkenhorst *et al.*, 2013). Whilst culture is considered the gold standard of microbial detection, it is also labour intensive, costly, and time-consuming: with *Salmonella* sp., due to enrichment and isolation steps, 3 days are required to confirm samples as salmonella negative and longer to confirm presumptive isolates (Farrell *et al*., 2005; Cheung and Kam, 2012; Falkenhorst *et al.*, 2013; Verdoodt *et al*., 2017; Vinayaka *et al*., 2018; Mobed *et al.*, 2019).

Skladal (2019) noted that pathogen detection time is critical to control the spread of infection and to apply immediate treatment. Rapid detection for *Salmonella* sp. is required to significantly enhance

diagnosis and treatment efficiency, as well as reduce resource use, and to provide reliable, cheap and effective screening for epidemiological studies (Kingsley *et al.,* 2009; Cheung and Kam, 2012).

Rapid detection methods that enable point of care testing are also desirable, enabling continuous herd screening, quick countermeasures for infectious disease, and therefore potentially avoiding farm-wide contamination (Ewald *et al*., 2013). Point of care testing is an 'on site' test, completed at the site of infection (Holford *et al.*, 2012). Zhu *et al*. (2019) note that the global need for point of care testing is expanding continuously. The World Health Organisation (WHO) developed the 'ASSURED' criteria for point of care testing, with tests being Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, Deliverable to the end user (Peeling *et al.*, 2006).

Thus, rapid diagnostics for *Salmonella* detection should be as specific and sensitive as conventional culture methods (Silva *et al.*, 2011; Mobed *et al.*, 2019). Additionally, in the case of farms with large herds of animals, cost per test is important, to reduce economic strain and to ensure effective treatment is delivered (Ewald *et al*., 2013). Rapid diagnostics that do not require expensive, sophisticated apparatus, or trained professionals to complete the test, would reduce costs and allow for 'on site' testing (Mobed *et al.*, 2019; Zhu *et al*., 2019). Reliability is essential to allow samples to be rapidly screened, with positive samples being confirmed by culture and negative samples dismissed with confidence (Moore and Feist, 2007). Non-invasive target samples, such as sweat, saliva, or faecal matter, would be ideal to ensure minimal distress to the infected animal (Holford *et al.*, 2012).

Moore and Feist (2007) state that the need for rapid detection methods for *Salmonella* sp. is generated by the widespread problems caused by the disease, however, due to the diversity of the organism, it is difficult to develop methods that can detect every *Salmonella* serotype. Therefore, one important criterion for the development of rapid salmonellae diagnostics is the ability to detect all serotypes (Moore and Feist, 2007).

Within this study, two types of rapid pan-*Salmonella* detection methods were developed, established and tested, to allow for point of care detection of salmonellosis in calves through scour. Tests were required to be highly specific and sensitive, robust to abrasive conditions and contaminates, quick, user-friendly, with simple, easily interpretable results.

The first assay, a molecular test targeting and amplifying the genomic DNA of *Salmonella* sp., utilised loop-mediated isothermal amplification. Bioinformatic techniques were deployed to align 12 complete *Salmonella* genomes to identify highly conserved pan-*Salmonella* genes to target for molecular detection. A partial screening of the alignment identified 32 conserved genes across the *Salmonella* genomes, 11 of which were considered highly specific to multiple *Salmonella* strains. Three of the highly specific genes were chosen to be used in developing molecular amplification assays; *bapA*, *hilA*, and *orgA.*

Two of the target genes, *hilA* and *orgA*, are associated with Salmonella Pathogenicity Island 1 (SPI 1), a conserved area coding for virulence phenotypes (Hensel, 2004). SPI1 is present on all subspecies and serotypes of *S. enterica* and *S. bongori* that have been analysed so far making it an excellent target for molecular detection (Hensel, 2004). SPI1 is reported to be highly regulated by two genes, one of which is *hilA*, which regulates the expression of several invasion genes including *orgA* (Altier, 2005; Galan, 1996; Mills *et al*, 1995). Coded within SPI 1, *orgA* is associated with the formation of type III secretion needle structure, which enables *Salmonella* sp. invasion (Klein *et al*., 2000; Kubori *et al.*, 1998).

The other gene targeted within this study was *bapA*, which is associated with biofilm formation potentially promoting cell-cell interactions (Latasa *et al*., 2005). Biswas *et al*. (2010) found that *bapA* could be used in PCR to successfully detect 34 different *Salmonella* serotypes.

Polymerase chain reaction (PCR) is considered the gold standard in molecular detection due to high sensitivity and specificity. Within this study, PCR primers were generated to allow for experimental detection of the genes identified as targets by bioinformatics. Using bioinformatics, two sets of PCR

primers were generated per target gene: six total. This was a simple process due to PCR being an established technique, with good supporting software for primer generation and that PCR primer sets only require two primers per set, forward and reverse.

Conventional PCR has been reported to be time-consuming, labour intensive, complex, and expensive, which supports the findings of this study (Cheung and Kam, 2012; Verdoodt *et al*., 2017; Mobed *et al*., 2019; Kaneko *et al*., 2006; Notomi et al., 2000; Parida et al., 2008; Mori et al., 2001). Whilst primer generation was simple, PCR protocol establishment was not and required troubleshooting. However once established and optimised, all PCR primer sets generated detected *S.* Dublin DNA, showing that *bapA, hilA* and *orgA* could be targeted for the detection of *S*. Dublin. When tested against multiple *Salmonella* serovars, only orgA_1 PCR primer set identified all tested salmonellae, however 5 primer sets recognised at least four of the six *Salmonella* serovars tested. There are several potential reasons for this.

Possibly, due to the limited number of complete genomes found on the NCBI database at the time of collection (Jan 2015), the targeted genes were not as specific as originally thought. However, when identified, genes were screened for *Salmonella* sp. specificity through BLASTn and only considered highly specific if they recognised multiple *Salmonella* serovars with complete to 99% sequence coverage. Within the PCR experiments, the *Salmonella* strains used were wildtype isolates. Potentially, single base-pair differences at the 3' end of the primers existed within the primer target DNA and resulted in reduced PCR primer efficacy and thus a lack of amplification. If primer efficacy was reduced, due to base-pair changes or sub-optimal cycling conditions, an increased concentration of *Salmonella* template DNA or additional cycles, could have improved amplification resulting in the PCR primer sets detecting all the *Salmonella* strains tested.

As developing an effective and reliable PCR assay to detect *Salmonella* sp. was not the aim of this study, additional optimisation was considered unnecessary, but future work could look at developing a robust PCR method utilising the PCR primer sets generated.

Once it had been determined that *S*. Dublin DNA could be reliably detected using molecular amplification by targeting *bapA*, *hilA,* and *orgA*, LAMP primer sets were generated. Loop-mediated Isothermal Amplification (LAMP) uses 6 primers designed to specifically target 8 distinct regions on the target gene to allow for nucleic acid amplification by DNA polymerase-mediated strand displacement activity at a constant temperature (Parida *et al*., 2008). Reported to be highly specific with highly efficient amplification, LAMP is a rapid technique that can be adapted easily to on-site testing (Mori *et al.,* 2001).

To enable LAMP assay development, LAMP primers were generated targeting *bapA*, *hilA*, and *orgA*. Due to LAMP requiring 6 primers, as opposed to the 2 needed for PCR, LAMP primer design was more complex and, potentially as LAMP is a relatively new technique first reported by Notomi *et al.* (2000), the supporting software (Primer Explorer V. 4) for primer development was not user-friendly. Despite this, 8 LAMP primer sets were generated, two for *hilA* and *orgA*, and four for *bapA*. Primer Explorer V. 4 only accepted base-pair sequences of 2,000bp, thus the sequence for *bapA* was segmented and two sections of the gene were targeted for primer development.

Initial development of the LAMP assay was successful, with clear visualisation of ladder pattern associated with LAMP amplicon on agarose gels after electrophoresis for all primer sets except bapA1.2, which was subsequently screened out. As with rapid detection methods it is important for them to be as quick and simple as possible without losing reliability, visualisation techniques that would eliminate the need for post-amplification electrophoresis were sought.

Initially turbidity of the reaction mixture was investigated. Despite others reporting success with visual turbidity (Mori *et al*., 2001), this study did not find clear turbidity in test assays when compared to control assays. Visualisation utilising colorimetric dyes was then explored, using hydroxy naphthol blue, methylene Blue, Nile Blue A and propidium iodide.

Colorimetric dyes were originally added to the reaction mixture before amplification, however as most dyes were DNA intercalating, they reacted with template DNA, resulting in no amplification. Thus, dyes were then added after amplification. Hydroxyl naphthol blue and methylene blue were screened out, as discussed in section 5.4. Whilst success was seen with Nile Blue A and propidium iodide colorimetric visualisation of LAMP test assays compared to control assays, the colour change was faint and subjective. For point of care rapid diagnostics, easily interpreted results are needed to allow for decisive action in terms of diagnosis and treatment. Thus, fluorometric visualisation was investigated using propidium iodide, SYBR safe, and SYBR Green I, under a UV light.

All fluorescent dyes showed clear, definitive visual difference between test and control assays, when under UV light. Due to this success, SYBR Green I was screened out, despite working well, due to the expense of the dye compared the others tested.

LAMP assay amplification time was shortened from an hour to 30 mins and increased sensitivity to low levels of amplification was observed with propidium iodide compared to SYBR safe during these experiments. At 30 minutes, low amplification was seen with bapA1.1, bapA1.2, bapA2.2, and hilA1 primer sets compared to that of bapA2.1, hilA2, orgA1, and orgA2, and thus bapA1.1, bapA1.2, bapA2.2, and hilA1 were screened out of the study.

The temperature range of the LAMP assay was tested, with different primer sets performing better at lower temperatures than others. However, 65°C was the optimum temperature for visualisation with propidium iodide for most primer sets. When the optimised fluorometric LAMP assay, 30 mins amplification at 65°C, was tested against a panel of *Salmonella* serovars, bapA2.1 and orgA1 detected all those screened. Except *S.* Mbandaka, orgA2 detected all salmonellae screened.

Before challenging the assay with scour, cross-over contamination from LAMP amplicon was observed. Despite a stringent contamination removal protocol, contamination could not be eliminated during this study. However, with future work to combine UDG digestion into the current protocol, this

contamination could be easily eliminated (Hsieh *et al*., 2014). Alternatively, adapting the current assay by utilising hydroxy naphthol blue or calcein into an all-in-one reaction tube in an area free of LAMP cross-over contamination, would also work and improve the ease of the overall protocol (Goto *et al*., 2009; Tomita *et al.*, 2008; Parida *et al*., 2008).

The optimised LAMP assay developed in this study can be completed and visualised in under 40 minutes using minimal apparatus, that can be purchased both affordably and battery operated, to detect pan-*Salmonella*.

The second rapid detection method tested within this study was a potentiometric immunoassay utilising biosensors and the Vantix system 2.0 (VR2). Reported as a highly specific, highly sensitive, rapid, and cheap, electrical biosensors measure the change in potential of an assay (Bahadir and Sezginturk, 2015; Fei *et al.*, 2015; Holford *et al*., 2012; Felix and Angnes, 2018). Based on antigen-antibody interactions, immunoassays are widespread in clinical diagnosis, with Enzyme-linked Immuno-sorbent assays (ELISA) considered the gold standard (Zhu *et al.*, 2019; Mobed *et al.,* 2019; Holford *et al*., 2012). Despite this, ELISA is a laboratory intensive method that takes approximately 4-6 hours (Danckert *et al.,* 2014). Additionally, immunoassays can require a pre-enrichment step, 16-20hrs, to allow for detection (Cheung and Kam, 2012).

To establish an immunoassay to detect pan-*Salmonella* using the Vantix system, ELISAs were used to determine antibody specificity and to develop a sandwich assay for adaption. Commercially available antibodies targeting the somatic (O) antigens of *Salmonella* serovars were selected and a direct ELISA assay was established to determine detection of a selection of *Salmonella* serovars. After protocol optimisation, washing plates with a wash bottle and incubating antibody steps at 37°C were found to give the best signal generation with the least deviation between data sets. It was determined that the polyclonal antibody (TRP) from Thermofisher would be the best capture antibody with conjugated monoclonal antibody (A99H) from Thermofisher as the detection antibody would be utilised in the sandwich immunoassay using the Vantix system.

The Vantix system allows for detection of the change in voltage between a test and reference probe. The test probe acts as a reaction surface, which the sandwich immunoassay occurs on. Simple, practical, and cost-effective, the Vantix system allows for the adaption of existing ELISA protocols, using the same reagents, to achieve the same sensitivity and specificity (Purvis *et al*., 2003; Stead *et al*., 2011; Cork *et al*., 2012).

The original Vantix reader (VR1) was used initially to establish that the ELISA protocol generated within this study could be adapted with good signal difference between control and test probes. Additionally, direct immunoassays were tested to whether reduction in antibody incubation time would affect signal production. Good signal production was seen at reduced time points, suggesting that the Vantix sandwich assay could undergo time reductions without effecting signal production. The VR1 had operational issues that were largely solved by utilising the Vantix reader 2.0 (VR2).

Potentiometric immunoassays using the VR2 looked at reducing the overall incubation time of the assays as well as optimising monoclonal antibody concentration. Once probes were prepared, results can be read and interpreted at 30 seconds into reading. A99H produced stronger signals in the presence of *Salmonella* at a concentration of 1:100, as opposed to the 1:500 used before. Overall incubation time was reduced from 2.5 hours to 1.25 hours. At 1.25 hours overall incubation, *S.* Dublin could be detected down to $2.13x10^7$ cfu/ml. Whilst overall incubation times of 1 hour, and 40 minutes were tested, a reduction in the efficacy of *Salmonella* detection was seen. When tested against different *Salmonella* serovars, the potentiometric immunoassay generated a greater signal for all serovars, above that of the control probe *E. coli* at 30 seconds, after the probe is exposed to substrate. In 1.25 hours overall incubation time, using commercial antibodies, the optimised Vantix immunoassay can detect pan-*Salmonella* with easily discernible results. With antibodies raised to be highly specific to pan-*Salmonella*, it is likely that this overall incubation time could be reduced further without losing the specificity to *Salmonella* serovars, whilst increasing the sensitivity of the assay.

Salmonella sp. are shed in the faecal matter of those infected, thus this makes it an excellent target for the detection of salmonellae in calves with scour, with minimal distress caused to the animal (Nielsen, 2013; Jadidi *et al*., 2012). However, calf scour is often acidic due to the milk diet of calves and inflammation of the calf bowel and contains gastroenteric bacteria as well as digested matter as competing factors for detection. Due to this most detection methods require prior sample preparation to separate target organism.

Spiked scour samples were used to replace bacterial suspensions within the optimised immunoassay on the VR2. Bacterial concentrations were a 1:10 dilution of the concentrations used before; a muted response was expected, however a clear difference in signal production was seen between *S.* Dublin compared *E. coli* down to a bacterial concentration 4.07×10^6 cfu/ml. Using commercial antibodies, the potentiometric immunoassay developed in this study can detect *S*. Dublin through undiluted scour.

Despite this, it was decided that a 1:2 dilution of the scour could potentially improve the signal produced in the presence of *S.* Dublin. The signal produced in the presence of *S*. Dublin in diluted scour was greater than that seen through undiluted scour. Scour is a known antagonistic sample: signal increase is likely due to reduced interference that could potentially be due to the reduction of unspecific binding by particulates that could compete with specific *S.* Dublin binding.

With probes pre-prepared to receive faecal samples, the current immunoassay using the VR2 could detect *S*. Dublin through calf scour in 45 minutes, on par with the Vantix immunoassay created by Stead *et al*. (2011) to detect Tylosin in feed. The sensitivity of an immunosensor is reported to be strongly connected to the affinity of the antibody to antigen: by raising more specific antibodies it is likely that the signal generated through faecal matter will be vastly improved (Fei *et al*., 2015; Haji-Hashemi *et al*., 2019; Purvis *et al*., 2003).

Cork *et al.* (2012) noted that repeatability of Vantix assays could be improved via automated production or robotic pipetting: hand pipetting small volumes (3µl) can be prone to error. Applicable

to the LAMP assay, this could also be reduced with the use of an electrical or stepper pipette, reducing human error to reduce the variation between data sets and increase reliability.

Vantix immunoassays would be vastly cheaper than ELISAs at a per-test cost level, due to the small aliquots of reagents needed to generate a signal. In commercialised biosensor kits, probes come pre-prepared, further reducing detection times. Additionally, due to the electronic numerical data produced by VR2, this would be suitable for transmission via mobile networks, allowing for remote disease control (Cork *et al.*, 2012). Vantix is commercially available and reasonably priced compared to other point of care systems. Additionally, the VR2 uses an established screen-printing technology, offering the prospect of cheap mass production (Cork *et al.*, 2012).

However, antibodies can have a limited shelf life, with batch to batch variation and often require cold storage (Wu *et al.*, 2014). Due to this, it unlikely that the Vantix system will be feasible for use in developing countries. Whilst easy to use, the Vantix protocol does require some level of pipetting skill, due to the small aliquots of reagents. The VR2 can be powered via a USB, with results visualised on a laptop, however completing the required steps on site might be unfeasible.

It would be recommended that the VR2 would be an excellent system for veterinarians, who already possess basic laboratory skills, as either a point of care system, or as a clinic detection system, allowing veterinarians to forgo sending samples to centralised labs and speeding up diagnostic and treatment intervals. Without the lengthy sample preparation steps associated with *Salmonella* diagnostics, the Vantix is a reliable, robust biosensor that can detect multiple *Salmonella* serovars through calf scour.

The fluorometric LAMP assay developed within this study has potential to initially be cheaper than the Vantix immunoassay, due to the initial cost of the VR2 itself. However, due to the sensitivity of LAMP, a true positive result may not be of clinical significance, as the detected DNA could be from dead or degrading microorganisms (Borst *et al.*, 2004). Additionally, the LAMP assay still needs to be challenged through faecal matter, research that was planned but not achieved within this study.

However due to the extremely high specificity of LAMP, due to the primers targeting six distinct regions of the template DNA and amplifying a specific gene with discrimination down to a single nucleotide difference, there is confidence that LAMP is robust enough to detect *Salmonella* sp. through calf scour (Mori *et al*., 2001; Parida *et al*., 2008; Tomita *et al.*, 2008). Notomi *et al*. (2000) found that LAMP not only had a high efficiency but is not significantly influenced by non-target DNA within the reaction assay with Francois *et al.* (2011) noting that LAMP remained highly robust and sensitive through impure samples, including faeces and urine.

LAMP is easily adaptable for field conditions, with simple operation, easy naked eye monitoring and cost-effective reaction equipment, it is a practical technique for low resource settings (Parida *et al*., 2008). Saffie *et al.* (2014) utilised a compact, portable heating block that can be used wherever 12V power was available and suggest that point-of-care testing could be achieved by using a rechargeable heating block and thermostabilised reagents.

It would be recommended that the LAMP assay developed within this study would be best suited to point of care testing, particularly on farm or in low resource settings, such as in developing countries. Due to the potential for simple sample addition and easily interpreted results, a skilled professional would not be needed to operate this assay for pan-*Salmonella* detection.

Cheung and Kam (2012) note that rapid methods for *Salmonella* detection would significantly reduce the resources required in routine laboratory operations, enhancing overall efficiency and productivity of public health laboratory services. By utilising either of the rapid detection methods developed within this study at the point of sample delivery, time and resources could be significantly reduced by screening out *Salmonella* negative samples and only culturing presumptive samples for confirmation. In the case of negative samples, this would allow *Salmonella* infection to be ruled out immediately, allowing for quicker diagnosis of other causal agents.

Early and accurate recognition of infected animals plays an important role in infection control programmes and disease eradication (Madi *et al*., 2012). Routine sampling for environmental and public safety purposes is commonplace to detect contamination increases and determine future actions, therefore precision and accuracy are important (Holford *et al*., 2012). The robust and reliable rapid diagnostics developed here, would facilitate the screening and sampling of *Salmonella* sp. to enable disease control, allowing monitoring of *Salmonella* sp. intra-herd, inter-herd, and on a national level. By utilising on site diagnostics, continuous herd screening and quick counter measures could be employed to avoid the following contamination of the production site, quicker that sending samples to centralised laboratories (Ewald *et al*., 2013).

The potentiometric immunoassay for the detection of *Salmonella* serovars developed for the VR2, rapid, completed and read in under an hour, and is robust through calf scour. With the potential for cheap, easy biosensor mass production and the adaptability of using established ELISA protocols, the VR2 shows great promise as a rapid detection system that could easily be immediately utilised. Whilst future research is needed to ensure robustness through scour, the fluorometric LAMP assay is quick and simple, with visible results generated in 40 minutes. With high specificity and sensitivity, LAMP shows promise as a detection method for *Salmonella* serovars on site, pen-side to infected cattle. Overall, two promising, rapid detection methods, capable of detecting multiple *Salmonella* serovars under 45 minutes have been developed both with advantages as point of care tests, including simple to use, with easily interpretable results.

9. Appendices

Appendix 1: The complete LAMP primer sets and their specifications

Gene target	LAMP Primer set used	Loop primer set used	Dimer potential (ΔG)	Primer type	5' pos	3' pos	Primer length (bp)	Tm (°C)	5' ΔG	3' ΔG	GC rate	Sequence	Base pair no. targeted by primer set (bp)
hilA	**22**		-0.71	F3	82	102	21	56	-5.8	-4	0.43	CTACGCTCAGAAAAGAAAGTC	209
				B3	272	291	20	55.1	-4.2	-5.1	0.35	TGTTTCAATGTAACGATGCT	
				FIP			48					AAGGTGTTTTTACTCACAATCTCGC-AATATTCCGCCAAAAGAATATGC	
				BIP			39					GCGACGCGGAAGTTAACGAA-AGAATACGTCGTAAGGCAT	
				F2	103	125	23	57.6	-1.9	-4	0.35	AATATTCCGCCAAAAGAATATGC	
				F1c	155	179	25	61.9	-5	-6.3	0.4	AAGGTGTTTTTACTCACAATCTCGC	
				B2	239	257	19	55.2	-3	-5.6	0.42	AGAATACGTCGTAAGGCAT	
				B1c	197	216	20	62.9	-7.4	-4.9	0.55	GCGACGCGGAAGTTAACGAA	
		18	-1.6	LF	126	141	16	52	-4.7	-5.2	0.56	CAGGATGACCAGAACG	
				LB	220	234	15	51.2	-4.1	-6.6	0.53	TCTCTTACCCGCTGT	
	123		-0.62	F3	542	562	21	57.1	-8	-5.9	0.48	GCCCCGATTATTATATCTCCG	-
				B3	732	749	18	56.1	-5.1	-5.7	0.5	ATCTGCTTTGTGTCCCAG	
				FIP			43					GGTGATAACCTTTAACCCGAACTAT-GGCAGATGATACCCGATG	
				BIP			40					GCTGCACCAGGAAAGCATTAA-CTGAGCAAAAGATTCGCAA	
				F2	563	580	18	56.1	-6.1	-5.7	0.56	GGCAGATGATACCCGATG	
				F1c	607	631	25	60.4	-4.9	-3.1	0.4	GGTGATAACCTTTAACCCGAACTAT	
				B2	695	713	19	55.7	-5.6	-6.1	0.42	CTGAGCAAAAGATTCGCAA	
				B1c	633	653	21	61.1	-6.7	-2.9	0.48	GCTGCACCAGGAAAGCATTAA	
	321		-0.71	F3	85	105	21	56.3	-6.4	-4.1	0.38	CGCTCAGAAAAGAAAGTCAAT	206
				B3	272	291	20	55.1	-4.2	-5.1	0.35	TGTTTCAATGTAACGATGCT	
				FIP			45					TCCAGTAAGGTGTTTTTACTCACAA-ATTCCGCCAAAAGAATATGC	
				BIP			39					CGACGCGGAAGTTAACGAAG-AGAATACGTCGTAAGGCAT	
				F2	106	125	20	56.2	-5.2	-4	0.4	ATTCCGCCAAAAGAATATGC	
				F1c	161	185	25	60.4	-5.3	-4.6	0.36	TCCAGTAAGGTGTTTTTACTCACAA	
				B2	239	257	19	55.2	-3	-5.6	0.42	AGAATACGTCGTAAGGCAT	
				B1c	198	217	20	60.8	-7.4	-5.2	0.55	CGACGCGGAAGTTAACGAAG	
		30	-2.61	LF	126	142	17	56.2	-6.1	-5.2	0.59	GCAGGATGACCAGAACG	
				LB	220	234	15	51.2	-4.1	-6.6	0.53	TCTCTTACCCGCTGT	
orgA	**18**		-2.27	F3	87	104	18	60.4	-5.1	-5.1	0.56	TCCTCAGCGGTTGCAGAT	210
				B3	280	297	18	60.4	-6.3	-4.6	0.61	TGCCAGATCGGCTCTCAG	
				FIP			41					CTCCGTTCTTAAGCCGCCATGC-GCGCCGGAAATGATTGTCA	
				BIP			40					CTCACTGACGCAGCTGTGGC-TGGCAACCGAGTAAATACGC	

Gene target	LAMP Primer set used	Loop primer set used	Dimer potential (ΔG)	Primer type	5' pos	3' pos	Primer length (bp)	Tm (°C)	5' ΔG	3' ΔG	GC rate	Sequence	Base pair no. targeted by primer set (bp)
				F2	106	124	19	60.7	-8.7	-4.6	0.53	GCGCCGGAAATGATTGTCA	
				F1c	166	187	22	65.9	-6	-5.9	0.59	CTCCGTTCTTAAGCCGCCATGC	
				B2	256	275	20	60.1	-5.9	-5.3	0.5	TGGCAACCGAGTAAATACGC	
				B1c	207	226	20	65.4	-4.7	-6.4	0.65	CTCACTGACGCAGCTGTGGC	
		19	-3.3	LF	145	165	21	57	-7	-4.6	0.43	CGCCAGTATTAACTCATTTGC	
				LB	230	245	16	60.7	-4.9	-6	0.69	GTCAGTGGCGCCGACT	
	33		-2.27	F3	87	104	18	60.4	-5.1	-5.1	0.56	TCCTCAGCGGTTGCAGAT	-
				B3	281	298	18	60.1	-5.9	-4.6	0.56	TTGCCAGATCGGCTCTCA	
				FIP			41					CTCCGTTCTTAAGCCGCCATGC-GCGCCGGAAATGATTGTCA	
				BIP			40					ACTCACTGACGCAGCTGTGG-TGGCAACCGAGTAAATACGC	
				F2	106	124	19	60.7	-8.7	-4.6	0.53	GCGCCGGAAATGATTGTCA	
				F1c	166	187	22	65.9	-6	-5.9	0.59	CTCCGTTCTTAAGCCGCCATGC	
				B2	256	275	20	60.1	-5.9	-5.3	0.5	TGGCAACCGAGTAAATACGC	
				B1c	206	225	20	64.3	-4.9	-5.5	0.6	ACTCACTGACGCAGCTGTGG	
	42		-2.27	F3	87	104	18	60.4	-5.1	-5.1	0.56	TCCTCAGCGGTTGCAGAT	212
				B3	282	299	18	59	-5.9	-5.4	0.61	CTTGCCAGATCGGCTCTC	
				FIP			41					CTCCGTTCTTAAGCCGCCATGC-GCGCCGGAAATGATTGTCA	
				BIP			40					CTCACTGACGCAGCTGTGGC-TGGCAACCGAGTAAATACGC	
				F2	106	124	19	60.7	-8.7	-4.6	0.53	GCGCCGGAAATGATTGTCA	
				F1c	166	187	22	65.9	-6	-5.9	0.59	CTCCGTTCTTAAGCCGCCATGC	
				B2	256	275	20	60.1	-5.9	-5.3	0.5	TGGCAACCGAGTAAATACGC	
				B1c	207	226	20	65.4	-4.7	-6.4	0.65	CTCACTGACGCAGCTGTGGC	
		23	-3.01	LF	145	165	21	57	-7	-4.6	0.43	CGCCAGTATTAACTCATTTGC	
				LB	239	254	16	61.8	-7	-5.4	0.69	GCCGACTGCCGCAAGT	
*bapA*1	**153**		-1.15	F3	1490	1508	19	59.3	-4.5	-4.2	0.58	CTCAACGGAACGGGAGAAG	235
				B3	1706	1725	20	60.2	-3.6	-4.7	0.55	GTGATAACCGGCACATCTGG	
				FIP			37					CGCTTTGATCTACCGTGGCG-CGCCACGATCCGCATTC	
				BIP			38					GAGAGCAACGCGCACATCT-GCGTAAAGCCGTCCGAAGG	
				F2	1513	1529	17	60.2	-7.2	-4.9	0.65	CGCCACGATCCGCATTC	
				F1c	1555	1574	20	63.1	-5.7	-7.2	0.6	CGCTTTGATCTACCGTGGCG	
				B2	1667	1685	19	62.8	-5.4	-5.6	0.63	GCGTAAAGCCGTCCGAAGG	
				B1c	1607	1625	19	62.5	-5.4	-4.3	0.58	GAGAGCAACGCGCACATCT	
		8	-3.44	LF	1538	1553	16	52	-5.7	-6.3	0.5	AACCGATTTCTACGCC	
				LB	1631	1645	15	56.8	-6.3	-5.6	0.67	GCCGTAGCGACCGAT	
	478		-1.04	F3	844	862	19	60.4	-5.3	-4.4	0.58	AGTCCAGACGGTGGATGAC	213
				B3	1041	1057	17	60.7	-5.8	-4.1	0.65	CGTAGCCGGGCCGTTAT	
				FIP			38					CCAGGGTGCCATCGATATGATG-GCGCGTCGCCGGAATT	
				BIP			37					GTCGTTACGCTCAGTCCGG-CGCGCGATCGATAGCAAT	
				F2	871	886	16	62.5	-8.3	-4	0.69	GCGCGTCGCCGGAATT	
				F1c	914	935	22	62.6	-6.3	-4	0.55	CCAGGGTGCCATCGATATGATG	

Gene target	LAMP Primer set used	Loop primer set used	Dimer potential (ΔG)	Primer type	5' pos	3' pos	Primer length (bp)	Tm (°C)	5' ΔG	3' ΔG	GC rate	Sequence	Base pair no. targeted by primer set (bp)
				B2	1019	1036	18	59.7	-8.6	-4.8	0.56	CGCGCGATCGATAGCAAT	
				B1c	971	989	19	61.8	-5.3	-6.6	0.63	GTCGTTACGCTCAGTCCGG	
		25	-3.47	LF	889	906	18	60.3	-5.4	-6.6	0.61	ACGGTAGCGTAAGGGTCG	
				LB	991	1008	18	60.6	-5.2	-5.5	0.61	GCAAACCGATGGCGGTAC	
	556		-1.15	F3	1490	1508	19	59.3	-4.5	-4.2	0.58	CTCAACGGAACGGGAGAAG	-
				B3	1706	1725	20	60.2	-3.6	-4.7	0.55	GTGATAACCGGCACATCTGG	
				FIP			37					CGCTTTGATCTACCGTGGCG-CGCCACGATCCGCATTC	
				BIP			40					GAGAGCAACGCGCACATCTTT-GCGTAAAGCCGTCCGAAGG	
				F2	1513	1529	17	60.2	-7.2	-4.9	0.65	CGCCACGATCCGCATTC	
				F1c	1555	1574	20	63.1	-5.7	-7.2	0.6	CGCTTTGATCTACCGTGGCG	
				B2	1667	1685	19	62.8	-5.4	-5.6	0.63	GCGTAAAGCCGTCCGAAGG	
				B1c	1607	1627	21	63.6	-5.4	-3.4	0.52	GAGAGCAACGCGCACATCTTT	
bapA2	**43**		-1.29	F3	728	744	17	59.2	-7.5	-4.9	0.65	CCGGCACCATCATCACC	221
				B3	934	949	16	61.1	-5.3	-5.4	0.69	TTAGCGGCGCGTCAGG	
				FIP			41					AACCCTTCGCTCAGATTACGGG-ACTGGCTACCGTCCAGGTC	
				BIP			37					ACGGATGCCGCAGGCAA-GGCTGGGTATCAAGGGTAAC	
				F2	765	783	19	62.8	-6.2	-5.4	0.63	ACTGGCTACCGTCCAGGTC	
				F1c	815	836	22	64.1	-5.3	-5.9	0.55	AACCCTTCGCTCAGATTACGGG	
				B2	904	923	20	59.4	-6.7	-4.3	0.55	GGCTGGGTATCAAGGGTAAC	
				B1c	859	875	17	64.2	-5.6	-5.8	0.65	ACGGATGCCGCAGGCAA	
		17	-1.9	LF	793	813	21	62.4	-6.1	-5.4	0.57	TAGCGGATAGGTCCAGCTACC	
				LB	883	899	17	61.5	-6.6	-4.6	0.65	CCGACCTCCGGCGTTTT	
	439		-1.55	F3	1463	1479	17	59.6	-5.7	-5.4	0.65	CCCTGACTGCCATTGCC	230
				B3	1674	1693	20	59.1	-6.6	-5.2	0.5	CGCCATTGTCGTAAATCGTG	
				FIP			38					GAACGGTGTCGACGGTGAAGG-GATGCCGCCGGAAACAG	
				BIP			35					TTGCACCAGTGACCGGGC-TTCGCCGCTGCCGTTAA	
				F2	1483	1499	17	60.2	-5.8	-4.2	0.65	GATGCCGCCGGAAACAG	
				F1c	1523	1543	21	64.9	-5.8	-4.9	0.62	GAACGGTGTCGACGGTGAAGG	
				B2	1643	1659	17	61.5	-6.5	-4.2	0.59	TTCGCCGCTGCCGTTAA	
				B1c	1583	1600	18	64.7	-5.6	-8	0.67	TTGCACCAGTGACCGGGC	
		22	-3.18	LF	1500	1518	19	63.1	-5.4	-7	0.63	GCTGTTCGATACGCCGCTG	
				LB	1604	1627	24	64.8	-5	-4.5	0.5	TAACCGATGGCGCCTTTACTAACG	

Appendix 2: The non-specific matches to each primer within the *bapA*1_1 LAMP set. Blue represents the forward primer set, yellow represents that backwards primer set and matches are highlighted in green.

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
Arabidopsis thaliana chromosome 1 BAC F14C21 genomic sequence, complete sequence	Achromobacter xylosoxidans C54, complete genome	Aggregatibacter actinomycetemcomitans HK1651, complete genome	Aeromonas schubertii strain WL1483, complete genome	Achlya hypogyna isolate S_ACHHYP_15881 secreted protein gene, complete cds	Acetobacter pasteurianus 386B, complete genome
Arabidopsis thaliana chromosome 1 sequence	Achromobacter xylosoxidans genome assembly NCTC10807, chromosome : 1	Aggregatibacter actinomycetemcomitans NUM4039 DNA, complete genome	Angiostrongylus cantonensis genome assembly A_cantonensis_China, scaffold ACAC_contig0002118	Advenella mimigardefordensis DPN7, complete genome	Acetobacter pasteurianus NBRC 101655 DNA, complete genome
Arabidopsis thaliana chromosome I BAC T24C10 genomic sequence, complete sequence	Achromobacter xylosoxidans NH44784-1996 complete genome	Angiostrongylus cantonensis genome assembly A_cantonensis_China, scaffold ACAC_scaffold0003907	Aspergillus clavatus NRRL 1 conserved hypothetical protein (ACLA_020530), partial mRNA	Amycolatopsis japonica strain MG417-CF17, complete genome	Aggregatibacter actinomycetemcomitans ANH9381, complete genome
Arabidopsis thaliana Full-length cDNA Complete sequence from clone GSLTPGH93ZH12 of Hormone Treated Callus of strain col-0 of Arabidopsis thaliana (thale cress)	Achromobacter xylosoxidans strain FDAARGOS_150, complete genome	Aureobasidium subglaciale EXF-2481 hypothetical protein partial mRNA	Azoarcus sp. CIB, complete genome	Amycolatopsis orientalis HCCB10007, complete genome	Aggregatibacter actinomycetemcomitans D11S-1, complete genome
Arabidopsis thaliana hypothetical protein (At1g54955) gene, complete cds	Aeromonas hydrophila YL17, complete genome			Angiostrongylus cantonensis genome assembly A_cantonensis_China, scaffold ACAC_contig0000166	Aggregatibacter actinomycetemcomitans HK1651, complete genome
Arabidopsis thaliana hypothetical protein (At1G54955) mRNA, complete cds	Arthrobacter sp. YC-RL1, complete genome			Angiostrongylus costaricensis genome assembly A_costaricensis_Costa_Rica, scaffold ACOC_contig0001227	Aggregatibacter actinomycetemcomitans HK1651, complete genome
Archaeoglobus veneficus SNP6, complete genome	Aspergillus terreus NIH2624 hypothetical protein (ATEG_08183) partial mRNA			Apteryx australis mantelli genome assembly AptMant0, scaffold scaffold55	Arthroderma benhamiae CBS 112371 hypothetical protein, mRNA
Aspergillus niger clone AXAS73-F19, complete sequence					
Aspergillus niger contig An11c0320, genomic contig					
Burkholderia cepacia strain LO6, complete genome	Bordetella bronchiseptica DNA, complete genome, strain: S798		Bacillus smithii strain DSM 4216, complete genome	Bacteroides fragilis genome assembly BFBE1.1, chromosome : scaffold1	Bacillus sp. genome assembly BS34ACh, chromosome : I
Burkholderia dolosa AU0158 chromosome 1, complete sequence	Bordetella bronchiseptica MO149 complete genome		Bifidobacterium breve strain BR3, complete genome	Bacteroides fragilis strain BOB25, complete genome	Bacillus sp. LM 4-2, complete genome
Burkholderia dolosa AU0158 chromosome 2, complete sequence	Bordetella bronchiseptica strain ATCC:BAA-588D-5, complete genome		Bosea sp. PAMC 26642, complete genome		Bacillus sp. YP1, complete genome
Burkholderia dolosa AU0158 chromosome 3, complete sequence	Bordetella bronchiseptica strain RB50, complete genome; segment 12/16		Burkholderia cenocepacia MC0-3 chromosome 3, complete sequence		Bacillus subtilis BEST7003 DNA, complete genome
Burkholderia fungorum strain ATCC BAA-463 chromosome 1, complete sequence	Bordetella parapertussis Bpp5 complete genome		Burkholderia cepacia GG4 chromosome 2, complete sequence		Bacillus subtilis BEST7613 DNA, complete genome
Burkholderia fungorum strain ATCC BAA-463 chromosome 3, complete sequence	Bordetella parapertussis strain 12822, complete genome; segment 11/14				Bacillus subtilis genome assembly BS49Ch, chromosome : I
Burkholderia oklahomensis C6786 chromosome I, complete sequence	Bordetella pertussis 137, complete genome				Bacillus subtilis HJ5, complete genome
Burkholderia oklahomensis C6786 chromosome II, complete sequence	Bordetella pertussis 18323 complete genome				Bacillus subtilis KCTC 1028, complete genome
Burkholderia oklahomensis strain EO147 chromosome 1, complete sequence	Bordetella pertussis B1917, complete genome				Bacillus subtilis PY79, complete genome
Burkholderia oklahomensis strain EO147 chromosome 2, complete sequence	Bordetella pertussis B1920, complete genome				Bacillus subtilis QB928, complete genome
Burkholderia phymatum STM815 chromosome 2, complete sequence	Bordetella pertussis CS, complete genome				Bacillus subtilis strain PS832, complete genome
Burkholderia sp. KJ006 chromosome 3, complete sequence	Bordetella pertussis genome assembly BPD420, chromosome : 1				Bacillus subtilis strain SG6, complete genome
Burkholderia thailandensis MSMB121 chromosome 2, complete sequence	Bordetella pertussis strain ATCC:BAA-1335D-5, complete genome				Bacillus subtilis strain TO-A JPC, complete genome

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
	Bordetella pertussis strain B1838, complete genome Bordetella pertussis strain B1865, complete genome Bordetella pertussis strain B3405, complete genome Bordetella pertussis strain B3582, complete genome Bordetella pertussis strain B3585, complete genome Bordetella pertussis strain B3621, complete genome Bordetella pertussis strain B3629, complete genome Bordetella pertussis strain B3640, complete genome Bordetella pertussis strain B3658, complete genome Bordetella pertussis strain B3913, complete genome Bordetella pertussis strain B3921, complete genome Bordetella pertussis strain H321, complete genome Bordetella pertussis strain I979, complete genome Bordetella pertussis strain Tohama I, complete genome; segment 8/12 Bordetella sp. N genome Bradyrhizobium sp. CCGE-LA001, complete genome Burkholderia ambifaria AMMD chromosome 2, complete sequence Burkholderia ambifaria AMMD chromosome 2, complete sequence Burkholderia ambifaria MC40-6 chromosome 2, complete sequence Burkholderia cenocepacia AU 1054 chromosome 1, complete sequence Burkholderia cenocepacia H111 chromosome 1 complete genome Burkholderia cenocepacia H111 chromosome 3, complete genome Burkholderia cenocepacia HI2424 chromosome 3, complete sequence Burkholderia cenocepacia J2315 chromosome 1, complete genome Burkholderia cenocepacia J2315 chromosome 3, complete genome Burkholderia cenocepacia MC0-3 chromosome 3, complete sequence Burkholderia cenocepacia strain K56-2 extracellular zinc metalloprotease precursor (zmpA) gene, complete cds Burkholderia cenocepacia strain LMG 16654 extracellular zinc metalloprotease precursor (zmpA) gene, complete cds Burkholderia cenocepacia strain LMG 18827 extracellular zinc metalloprotease precursor (zmpA) gene, complete cds Burkholderia cenocepacia strain LMG 18832 extracellular zinc metalloprotease precursor (zmpA) gene, complete cds				Bacillus subtilis strain UD1022, complete genome Bacillus subtilis subsp. natto BEST195 DNA, complete genome Bacillus subtilis subsp. natto strain CGMCC 2108, complete genome Bacillus subtilis subsp. subtilis 6051-HGW, complete genome Bacillus subtilis subsp. subtilis RO-NN-1, complete genome Bacillus subtilis subsp. subtilis str. 168, complete genome Bacillus subtilis subsp. subtilis str. AG1839, complete genome Bacillus subtilis subsp. subtilis str. BAB-1, complete genome Bacillus subtilis subsp. subtilis str. BSP1, complete genome Bacillus subtilis subsp. subtilis str. JH642 substr. AG174, complete genome Bacillus subtilis subsp. subtilis str. OH 131.1, complete genome Bacillus subtilis subsp. subtilis strain 3NA, complete genome Bacillus subtilis subsp. subtilis strain BSD-2, complete genome Bacillus subtilis subsp. subtilis strain CU1050, complete genome Bacillus subtilis TOA, complete genome Bacillus subtilis XF-1, complete genome

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
	Burkholderia cenocepacia strain ST32 chromosome 1, complete sequence Burkholderia cenocepacia strain ST32 chromosome 2, complete sequence Burkholderia cenocepacia strain ST32 chromosome 3, complete sequence Burkholderia cepacia ATCC 25416 chromosome 3, complete sequence Burkholderia cepacia ATCC 25416 chromosome 3, complete sequence Burkholderia cepacia extracellular zinc metalloprotease PSCP precursor (zmpA) gene, complete cds Burkholderia cepacia JBK9 chromosome 1, complete sequence Burkholderia cepacia JBK9 chromosome 3, complete sequence Burkholderia cepacia strain DDS 7H-2 chromosome 1, complete sequence Burkholderia cepacia strain DDS 7H-2 chromosome 3, complete sequence Burkholderia contaminans strain MS14 chromosome 3, complete sequence Burkholderia gladioli BSR3 chromosome 1, complete sequence Burkholderia gladioli strain DMSZ11318 putative regulator gene, partial cds; putative regulator gene, complete cds; and bongkrekic acid biosynthentic gene cluster, partial sequence				
Clostridium stercorarium subsp. stercorarium DSM 8532, complete genome Clostridium stercorarium subsp. stercorarium DSM 8532, complete genome Croceicoccus naphthovorans strain PQ-2, complete genome Cyanothece sp. ATCC 51142 circular chromosome, complete sequence Cyanothece sp. ATCC 51142 HupS (hupS) gene, complete cds; hupS-hupL intergenic spacer, complete sequence; and HupL (hupL) gene, complete cds Cyanothece sp. BG04351 uptake hydrogenase small subunit (hupS) gene, partial cds Cyprinus carpio genome assembly common carp genome, scaffold 000001384	Cronobacter dublinensis subsp. dublinensis LMG 23823, complete genome	Corynebacterium stationis strain ATCC 6872, complete genome	Candidatus Accumulibacter phosphatis clade IIA str. UW-1, complete genome Candidatus Sodalis pierantonius str. SOPE, complete genome Celeribacter indicus strain P73, complete genome Citrobacter koseri genome assembly PRJEB6512_assembly_1, scaffold CONTIG000001 Cladophialophora carrionii CBS 160.54 hypothetical protein partial mRNA Corynebacterium humireducens NBRC 106098 = DSM 45392, complete genome Corynebacterium jeikeium K411 complete genome Cupriavidus necator N-1 chromosome 1, complete sequence Cylicostephanus goldi genome assembly C_goldi_Cheshire, scaffold CGOC_scaffold0015112	Cucumis melo genomic chromosome, chr_4 Cucumis melo genomic scaffold, anchoredscaffold00011	Citrobacter freundii strain CAV1321, complete genome Citrobacter freundii strain CAV1741, complete genome Corynebacterium kutscheri strain DSM 20755, complete genome
Desulfovibrio vulgaris DP4, complete genome Desulfovibrio vulgaris RCH1, complete genome	Desulfotomaculum acetoxidans DSM 771, complete genome Devosia sp. H5989, complete genome	Diphyllobothrium latum genome assembly D_latum_Geneva, scaffold DILT_contig0001764 Drosophila erecta uncharacterized protein (Dere\GG13502), mRNA	Deinococcus deserti VCD115, complete genome Desulfitobacterium dichloroeliminans LMG P-21439, complete genome	Deinococcus actinosclerus strain BM2, complete genome Deinococcus peraridilitoris DSM 19664, complete genome	Desulfovibrio alaskensis G20, complete genome

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
Desulfovibrio vulgaris subsp. vulgaris str. Hildenborough, complete genome Drosophila grimshawi GH17522 (Dgri\GH17522), mRNA Drosophila grimshawi GH20613 (Dgri\GH20613), mRNA		Drosophila yakuba uncharacterized protein, transcript variant A (Dyak\GE22287), mRNA Drosophila yakuba uncharacterized protein, transcript variant A (Dyak\GE23121), mRNA Drosophila yakuba uncharacterized protein, transcript variant B (Dyak\GE22287), mRNA Drosophila yakuba uncharacterized protein, transcript variant B (Dyak\GE23121), mRNA	Dyella jiangningensis strain SBZ 3-12, complete genome	'Deinococcus soli' Cha et al. 2014 strain N5, complete genome Drosophila virilis uncharacterized protein (Dvir\GJ10640), mRNA	
Echinostoma caproni genome assembly E_caproni_Egypt, scaffold ECPE_scaffold0011806 Echinostoma caproni genome assembly E_caproni_Egypt, scaffold ECPE_scaffold0015965	Emiliania huxleyi CCMP1516 hypothetical protein partial mRNA Emiliania huxleyi CCMP1516 hypothetical protein partial mRNA	Echinostoma caproni genome assembly E_caproni_Egypt, scaffold ECPE_scaffold0034562 Enterococcus faecium isolate EFE10021 genome assembly, chromosome: chr Enterococcus faecium strain 64/3, complete genome Enterococcus faecium strain 6E6, complete genome Enterococcus faecium strain UW7606x64/3 TC1, complete genome Escherichia coli ACN001, complete genome Escherichia coli B strain C2566, complete genome Escherichia coli B strain C3029, complete genome Escherichia coli genome assembly ERS742059, chromosome : I Escherichia coli K-12 GM4792 Lac-, complete genome Escherichia coli K-12 GM4792 Lac+, complete genome Escherichia coli K-12 strain C3026, complete genome Escherichia coli K-12 strain DHB4, complete genome Escherichia coli O157:H7 strain WS4202, complete genome Escherichia coli PCN033, complete genome Escherichia coli PCN061, complete genome Escherichia coli str. K-12 substr. MG1655 strain JW5437-1, complete genome Escherichia coli str. K-12 substr. MG1655, complete genome Escherichia coli str. K-12 substr. MG1655, complete genome Escherichia coli strain 2009C-3133, complete genome Escherichia coli strain 2012C-4227, complete genome Escherichia coli strain 268-78-1, complete genome Escherichia coli strain ACN002, complete genome Escherichia coli strain C43(DE3), complete genome Escherichia coli strain CD306, complete genome Escherichia coli strain CQSW20, complete genome	Enterobacter sacchari SP1, complete genome Escherichia coli strain MRE600, complete genome	Echinostoma caproni genome assembly E_caproni_Egypt, scaffold ECPE_scaffold0008043 Echinostoma caproni genome assembly E_caproni_Egypt, scaffold ECPE_scaffold0034927 Enterobacter cloacae strain GGT036, complete genome	Echinostoma caproni genome assembly E_caproni_Egypt, scaffold ECPE_scaffold0003724 Echinostoma caproni genome assembly E_caproni_Egypt, scaffold ECPE_scaffold0019480 Eggerthella sp. YY7918 DNA, complete genome Enterobius vermicularis genome assembly E_vermicularis_Canary_Islands, scaffold EVEC_scaffold0002012 Erythrobacter sp. s21-N3, complete genome Eubacterium limosum KIST612, complete genome

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
		Escherichia coli strain DH1Ec095, complete genome			
		Escherichia coli strain DH1Ec104, complete genome			
		Escherichia coli strain DH1Ec169, complete genome			
		Escherichia coli strain G749, complete genome			
		Escherichia coli strain JEONG-1266, complete genome			
		Escherichia coli strain JJ1897, complete genome			
		Escherichia coli strain JJ2434, complete genome			
		Escherichia coli strain K-12 substrain MG1655_TMP32XR1, complete genome			
		Escherichia coli strain K-12 substrain MG1655_TMP32XR2, complete genome			
		Escherichia coli strain MRE600, complete genome			
		Escherichia coli strain MVAST0167, complete genome			
		Escherichia coli strain NCM3722, complete genome			
		Escherichia coli strain RR1, complete genome			
		Escherichia coli strain RS76 genome			
		Escherichia coli strain SaT040, complete geome			
		Escherichia coli strain SEC470 genome			
		Escherichia coli strain SF-088, complete genome			
		Escherichia coli strain SF-166, complete genome			
		Escherichia coli strain SF-173, complete genome			
		Escherichia coli strain SF-468, complete genome			
		Escherichia coli strain ST648, complete genome			
		Escherichia coli strain uk_P46212, complete sequence			
		Escherichia coli strain YD786, complete genome			
		Escherichia coli strain ZH063, complete genome			
		Escherichia coli strain ZH193, complete genome			
		Exophiala xenobiotica hypothetical protein mRNA			
Fusarium fujikuroi IMI 58289 draft genome, chromosome FFUJ_chr01					Faecalibaculum rodentium strain Alo17, complete genome
Granulicella tundricola MP5ACTX9 plasmid pACIX901, complete sequence					
Heligmosomoides polygyrus genome assembly H_bakeri_Edinburgh, scaffold HPBE_contig0010452		Hymenobacter sp. DG5B, complete genome	Haloferax gibbonsii strain ARA6, complete genome		Heligmosomoides polygyrus genome assembly H_bakeri_Edinburgh, scaffold HPBE_scaffold0000167
Heligmosomoides polygyrus genome assembly H_bakeri_Edinburgh, scaffold HPBE_scaffold0002887		Hymenobacter sp. PAMC26628, complete genome	Halorhodospira halophila SL1, complete genome		Hymenobacter sp. PAMC26628, complete genome
Hordeum vulgare subsp. spontaneum voucher OUH602, partial sequence		Hymenolepis diminuta genome assembly H_diminuta_Denmark, scaffold HDID_contig0000029	Herbaspirillum hiltneri N3, complete genome		

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
		Hymenolepis diminuta genome assembly H_diminuta_Denmark, scaffold HDID_contig0000031			
	Inquilinus limosus anmk, blaINQ-1, dcpI, ycjk, oat and aat genes, strain MP06				
	Ketogulonicigenium vulgare strain Hbe602, complete genome Kocuria palustris strain MU14/1, complete genome		Kangiella koreensis DSM 16069, complete genome Kibdelosporangium phytohabitans strain KLBMP1111, complete genome	Kluyveromyces marxianus DMKU3-1042 DNA, complete genome, chromosome 2 Kluyveromyces marxianus DNA, chromosome 2, complete genome, strain: NBRC 1777 Kluyveromyces marxianus strain CCT 7735 (UFV-3) chromosome 2 sequence	
Leptolyngbya sp. NIES-3755 DNA, complete genome	Leucosporidium scottii MAT locus region, culture collection CBS:5930, contig node139	Lysobacter enzymogenes strain C3 genome Lysobacter gummosus strain 3.2.11, complete genome	Leishmania major strain Friedlin complete genome, chromosome 25 Leishmania major strain Friedlin conserved hypothetical protein (LMJF_25_0970) mRNA, complete cds Luteipulveratus mongoliensis strain MN07-A0370 genome Lysobacter antibioticus strain ATCC 29479 genome	Leishmania panamensis strain MHOM/PA/94/PSC-1 chromosome 35 sequence Lichtheimia ramosa genome assembly Lramosa_hybrid_454_Illumina, scaffold SCAF21	
Malassezia globosa CBS 7966 hypothetical protein MGL_4070 partial mRNA	Marssonina brunnea f. sp. 'multigermtubi' MB_m1 microtubule associated protein (MBM_06825), mRNA	Methanococcus maripaludis C5, complete genome	Magnaporthe oryzae 70-15 quinate permease (MGG_05600) mRNA, complete cds		Marinobacter hydrocarbonoclasticus str. ATCC 49840 chromosome, complete genome Meyerozyma guilliermondii ATCC 6260 hypothetical protein (PGUG_04352) partial mRNA
Melipona subnitida clone c63910_Msub11 microsatellite sequence	Martelella endophytica strain YC6887, complete genome	Methanococcus maripaludis C7, complete genome	Methanogenic archaeon mixed culture ISO4-G1, complete genome		
Mesocestoides corti genome assembly M_corti_Specht_Voge, scaffold MCOS_contig0000341	Massilia sp. NR 4-1, complete genome	Methanococcus maripaludis Glu-tRNA Gln amidotransferase subunit A (gatA) gene, partial cds; and conserved hypothetical protein, hypothetical protein, pyruvate oxidoreductase delta subunit (porA), pyruvate oxidoreductase gamma subunit (porB), pyruvate oxidoreductase alpha subunit (porC), pyruvate oxidoreductase beta subunit (porD), pyruvate oxidoreductase cysteine-rich subunit 1 (porE), pyruvate oxidoreductase cysteine-rich subunit 2 (porF), and conserved hypothetical protein genes, complete cds	Mycobacterium chubuense NBB4, complete genome		
Mouse DNA sequence from clone RP23-364B23 on chromosome 2, complete sequence Myxococcus hansupus strain mixupus, complete genome	Myxococcus hansupus strain mixupus, complete genome	Methanococcus maripaludis strain S2, complete sequence Methanococcus maripaludis X1, complete genome	Mycobacterium liflandii 128FXT, complete genome Mycobacterium marinum E11 main chromosome genome Mycobacterium marinum M, complete genome Mycobacterium tuberculosis strain 37004, complete genome Mycobacterium ulcerans Agy99, complete genome		

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
Neospora caninum Liverpool complete genome, chromosome II	Nectria haematococca mpVI 77-13-4 hypothetical protein, mRNA	Nitrosospira briensis C-128, complete genome	Novosphingobium pentaromativorans US6-1 plasmid pLA4, complete sequence		
Nippostrongylus brasiliensis genome assembly N_brasiliensis_RM07_v1_5_4, scaffold NBR_contig0000088		Nitrospira moscoviensis strain NSP M-1, complete genome	Novosphingobium sp. PP1Y main chromosome, complete replicon		
Nodularia spumigena CCY9414 genome					
		Oryza sativa Indica Group cultivar RP Bio-226 chromosome 1 sequence Oryza sativa Japonica Group DNA, chromosome 1, cultivar: Nipponbare, complete sequence		Octadecabacter antarcticus 307, complete genome	Oryza sativa Indica Group cultivar RP Bio-226 chromosome 9 sequence Oryza sativa Japonica Group DNA, chromosome 9, cultivar: Nipponbare, complete sequence
				Oplegnathus fasciatus signal transducer and activator of transcription 4 (STAT4) gene, complete cds	Ovis canadensis canadensis isolate 43U chromosome 11 sequence
Paracoccidioides brasiliensis Pb01 predicted protein, mRNA	Polymorphum gilvum SL003B-26A1, complete genome	Pandoraea apista strain AU2161, complete genome	Paenibacillus graminis strain BR304.07 RNA polymerase beta subunit (rpoB) gene, partial cds	Paenibacillus riograndensis SBR5 genome assembly SBR5(T), chromosome : I	PREDICTED: Bactrocera dorsalis protein three rows (LOC105222897), mRNA
Pelagibacterium halotolerans B2, complete genome	PREDICTED: Setaria italica transcription factor MYB44-like (LOC101754396), mRNA	Pandoraea apista strain DSM 16535, complete genome	Paenibacillus graminis strain MC04.06 RNA polymerase beta subunit (rpoB) gene, partial cds	Pandoraea apista strain AU2161, complete genome	PREDICTED: Bactrocera oleae protein three rows (LOC106620923), mRNA
Peregrinibacteria bacterium RIFOXYA2_FULL_PER-ii_58_14, complete genome	Pseudoalteromonas sp. Bsw20308, complete genome	Pirellula staleyi DSM 6068, complete genome	Paenibacillus graminis strain MC04.21 RNA polymerase beta subunit (rpoB) gene, partial cds	Pandoraea apista strain TF80G25, complete genome	PREDICTED: Esox lucius nudix (nucleoside diphosphate linked moiety X)-type motif 19 (nudt19), mRNA
Peregrinibacteria bacterium RIFOXYB2_FULL_PER-ii_58_17, complete genome	Pseudomonas syringae CC1557, complete sequence	Plasmodium vivax Sal-1 hypothetical protein partial mRNA	Paenibacillus graminis strain MC22.02 RNA polymerase beta subunit (rpoB) gene, partial cds	Pandoraea apista strain TF81F4, complete genome	PREDICTED: Fundulus heteroclitus ETS domain-containing protein Elk-3-like (LOC105916870), mRNA
Peregrinibacteria bacterium RIFOXYC2_FULL_PER-ii_58_32, complete genome	Pseudomonas syringae pv. lapsa strain ATCC 10859, complete genome	Pleurocapsa sp. PCC 7327, complete genome	Paenibacillus graminis strain MC22.12 RNA polymerase beta subunit (rpoB) gene, partial cds	PREDICTED: Anolis carolinensis SR-related CTD-associated factor 1 (scaf1), mRNA	PREDICTED: Trichogramma pretiosum slo-interacting protein 1 (LOC106652123), transcript variant X1, mRNA
Peregrinibacteria bacterium RIFOXYD1_FULL_PER-ii_59_16, complete genome	Pseudomonas syringae pv. syringae B301D, complete genome	PREDICTED: Beta vulgaris subsp. vulgaris auxin efflux carrier component 2 (LOC104908377), mRNA	Paenibacillus graminis strain MC36.22 RNA polymerase beta subunit (rpoB) gene, partial cds	PREDICTED: Bubalus bubalis collagen and calcium binding EGF domains 1 (CCBE1), transcript variant X1, mRNA	PREDICTED: Trichogramma pretiosum slo-interacting protein 1 (LOC106652123), transcript variant X2, mRNA
Peregrinibacteria bacterium RIFOXYD2_FULL_PER-ii_51_23, complete genome	Pseudomonas syringae pv. syringae B728a, complete genome		Paenibacillus graminis strain TOD221 RNA polymerase beta subunit (rpoB) gene, partial cds	PREDICTED: Bubalus bubalis collagen and calcium binding EGF domains 1 (CCBE1), transcript variant X2, mRNA	PREDICTED: Trichogramma pretiosum slo-interacting protein 1 (LOC106652123), transcript variant X3, mRNA
PREDICTED: Acromyrmex echinatior neuronal acetylcholine receptor subunit alpha-7-like (LOC105154928), transcript variant X1, mRNA	Pseudonocardia sp. AL041005-10, complete genome		Paenibacillus riograndensis SBR5 genome assembly SBR5(T), chromosome : I	PREDICTED: Cucumis melo uncharacterized protein At5g03900, chloroplastic (LOC103486612), mRNA	Pseudomonas monteilii SB3078, complete genome
PREDICTED: Acromyrmex echinatior neuronal acetylcholine receptor subunit alpha-7-like (LOC105154928), transcript variant X2, mRNA			Paenibacillus sp. FSL H7-0357, complete genome	PREDICTED: Dipodomys ordii natural killer cell triggering receptor (Nktr), mRNA	Pseudomonas monteilii SB3101, complete genome
PREDICTED: Acromyrmex echinatior neuronal acetylcholine receptor subunit alpha-7-like (LOC105154928), transcript variant X3, mRNA			Paenibacillus sp. FSL R7-0273, complete genome	PREDICTED: Larimichthys crocea signal transducer and activator of transcription 4 (stat4), mRNA	Pseudomonas putida strain DLL-E4, complete genome
PREDICTED: Cavia porcellus leucine rich repeat containing 71 (Lrrc71), transcript variant X1, mRNA			Paenibacillus sp. FSL R7-0331, complete genome	PREDICTED: Lingula anatina uncharacterized LOC106153658 (LOC106153658), mRNA	
PREDICTED: Cavia porcellus leucine rich repeat containing 71 (Lrrc71), transcript variant X2, mRNA			Paenibacillus typhae strain xj7 RNA polymerase beta subunit (rpoB) gene, partial cds	PREDICTED: Lingula anatina zinc finger protein 774-like (LOC106171778), mRNA	
PREDICTED: Cavia porcellus leucine rich repeat containing 71 (Lrrc71), transcript variant X3, mRNA			Pandoraea pnomenusa strain RB38, complete genome	PREDICTED: Monomorium pharaonis furin-like protease 2 (LOC105831356), transcript variant X1, mRNA	
PREDICTED: Cavia porcellus leucine rich repeat containing 71 (Lrrc71), transcript variant X4, mRNA			Pandoraea vervacti strain NS15, complete genome	PREDICTED: Monomorium pharaonis furin-like protease 2 (LOC105831356), transcript variant X2, mRNA	
PREDICTED: Cavia porcellus leucine rich repeat containing 71 (Lrrc71), transcript variant X5, mRNA			Planctomyces limnophilus DSM 3776, complete genome	PREDICTED: Notothenia coriiceps signal transducer and activator of transcription 4-like (LOC104961600), partial mRNA	

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
PREDICTED: Cavia porcellus leucine rich repeat containing 71 (Lrrc71), transcript variant X6, mRNA			PREDICTED: Austrofundulus limnaeus ATP-binding cassette, sub-family F (GCN20), member 2 (abcf2), mRNA	PREDICTED: Poecilia formosa zinc finger protein 362-like (LOC103136579), transcript variant X1, mRNA	
PREDICTED: Cavia porcellus leucine rich repeat containing 71 (Lrrc71), transcript variant X7, mRNA			PREDICTED: Camelina sativa uncharacterized LOC104750128 (LOC104750128), mRNA	PREDICTED: Poecilia formosa zinc finger protein 362-like (LOC103136579), transcript variant X2, mRNA	
PREDICTED: Cavia porcellus leucine rich repeat containing 71 (Lrrc71), transcript variant X8, misc_RNA			PREDICTED: Copidosoma floridanum endochitinase-like (LOC106639905), mRNA	PREDICTED: Poecilia latipinna zinc finger protein 362-like (LOC106956452), transcript variant X1, mRNA	
PREDICTED: Charadrius vociferus excision repair cross-complementation group 6-like (ERCC6L), mRNA			PREDICTED: Cynoglossus semilaevis baculoviral IAP repeat containing 6 (birc6), mRNA	PREDICTED: Poecilia latipinna zinc finger protein 362-like (LOC106956452), transcript variant X2, mRNA	
PREDICTED: Columba livia excision repair cross-complementation group 6-like (ERCC6L), mRNA			PREDICTED: Cyprinodon variegatus proline synthetase co-transcribed homolog (bacterial) (prosc), mRNA	PREDICTED: Poecilia mexicana zinc finger protein 362-like (LOC106926093), transcript variant X1, mRNA	
PREDICTED: Limulus polyphemus uncharacterized LOC106475716 (LOC106475716), ncRNA			PREDICTED: Gekko japonicus vacuolar protein sorting 4 homolog A (S. cerevisiae) (VPS4A), mRNA	PREDICTED: Poecilia mexicana zinc finger protein 362-like (LOC106926093), transcript variant X2, mRNA	
PREDICTED: Oryza sativa Japonica Group ras-related protein RABE1d (LOC4342644), mRNA			PREDICTED: Gekko japonicus vacuolar protein sorting-associated protein 4A (LOC107125207), partial mRNA	PREDICTED: Python bivittatus natural killer cell triggering receptor (NKTR), transcript variant X1, mRNA	
PREDICTED: Phoenix dactylifera isoflavone reductase-like protein (LOC103711540), mRNA			PREDICTED: Poecilia formosa ATP-binding cassette sub-family F member 2-like (LOC103149773), mRNA	PREDICTED: Python bivittatus natural killer cell triggering receptor (NKTR), transcript variant X2, mRNA	
PREDICTED: Strongylocentrotus purpuratus centrosomal protein of 95 kDa (LOC100888575), mRNA			PREDICTED: Poecilia formosa proline synthetase co-transcribed homolog (bacterial) (prosc), mRNA	PREDICTED: Python bivittatus natural killer cell triggering receptor (NKTR), transcript variant X3, mRNA	
PREDICTED: Tyto alba excision repair cross-complementation group 6-like (ERCC6L), partial mRNA			PREDICTED: Poecilia latipinna ATP binding cassette subfamily F member 2 (abcf2), transcript variant X1, mRNA	PREDICTED: Python bivittatus natural killer cell triggering receptor (NKTR), transcript variant X4, mRNA	
PREDICTED: Zea mays LOC100284072 (cl9715_1b), transcript variant X1, mRNA			PREDICTED: Poecilia latipinna ATP binding cassette subfamily F member 2 (abcf2), transcript variant X2, mRNA	PREDICTED: Sesamum indicum lisH domain and HEAT repeat-containing protein KIAA1468 homolog (LOC105177921), transcript variant X4, mRNA	
PREDICTED: Zea mays LOC100284072 (cl9715_1b), transcript variant X2, mRNA			PREDICTED: Poecilia latipinna proline synthetase co-transcribed homolog (bacterial) (prosc), mRNA	PREDICTED: Sesamum indicum lisH domain and HEAT repeat-containing protein KIAA1468 homolog (LOC105177921), transcript variant X5, mRNA	
PREDICTED: Zea mays LOC100284072 (cl9715_1b), transcript variant X3, mRNA			PREDICTED: Poecilia mexicana ATP binding cassette subfamily F member 2 (abcf2), transcript variant X1, mRNA	PREDICTED: Stegastes partitus signal transducer and activator of transcription 4 (stat4), transcript variant X1, mRNA	
PREDICTED: Zea mays LOC100284072 (cl9715_1b), transcript variant X4, mRNA			PREDICTED: Poecilia mexicana ATP binding cassette subfamily F member 2 (abcf2), transcript variant X2, mRNA	PREDICTED: Stegastes partitus signal transducer and activator of transcription 4 (stat4), transcript variant X2, mRNA	
			PREDICTED: Poecilia mexicana proline synthetase co-transcribed homolog (bacterial) (prosc), mRNA	PREDICTED: Trichogramma pretiosum LIM domain kinase 1 (LOC106659788), transcript variant X1, mRNA	
			PREDICTED: Poecilia reticulata proline synthetase co-transcribed homolog (bacterial) (prosc), mRNA	PREDICTED: Trichogramma pretiosum LIM domain kinase 1 (LOC106659788), transcript variant X2, mRNA	
			PREDICTED: Xiphophorus maculatus ATP-binding cassette, sub-family F (GCN20), member 2 (abcf2), transcript variant X1, mRNA	Propionibacterium freudenreichii subsp. freudenreichii genome assembly SURFING-batch1, scaffold Scaffold11	
			PREDICTED: Xiphophorus maculatus ATP-binding cassette, sub-family F (GCN20), member 2 (abcf2), transcript variant X2, mRNA	Propionibacterium freudenreichii subsp. freudenreichii strain DSM 20271, complete genome	

F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
			PREDICTED: Xiphophorus maculatus proline synthetase co-transcribed homolog (bacterial) (prosc), mRNA Protopolystoma xenopodis genome assembly P_xenopodis_South_Africa, scaffold PXEA_contig0071887 Protopolystoma xenopodis genome assembly P_xenopodis_South_Africa, scaffold PXEA_contig0120631 Pseudomonas fragi strain P121, complete genome Pseudomonas monteilii strain USDA-ARS-USMARC-56711, complete genome Pseudomonas sp. FGI182, complete genome	Pseudomonas knackmussii B13 complete genome	
Ralstonia pickettii DTP0602 chromosome 3, complete sequence	Rhizobium tropici CIAT 899, complete genome	Rhinocladiella mackenziei CBS 650.93 fumarylacetoacetase partial mRNA	Ralstonia pickettii DTP0602 chromosome 1, complete sequence	Rhodanobacter denitrificans strain 2APBS1, complete genome	
Rhizobium leguminosarum bv. trifolii WSM1325 plasmid pR132502, complete sequence	Rhodanobacter denitrificans strain 2APBS1, complete genome		Rhodothermus marinus DSM 4252, complete genome		
Rhizobium leguminosarum bv. viciae plasmid pRL11 complete genome, strain 3841	Rhodobacter sphaeroides ATCC 17025 plasmid pRSPA01, complete sequence		Roseiflexus sp. RS-1, complete genome		
	Rhodobacter sphaeroides strain MBTLJ-8 chromosome 1, complete sequence		Russula sp. TJ00/35 mitochondrial intermediate peptidase (mip) gene, partial cds; nuclear gene for mitochondrial product		
Strongylus vulgaris genome assembly S_vulgaris_Kentucky, scaffold SVUK_scaffold0023472	Sphingobium sp. MI1205 chromosome 1, complete sequence	Shigella boydii strain ATCC 9210, complete genome	Saccharomycetaceae sp. 'Ashbya aceri' chromosome III, complete sequence	Serratia liquefaciens ATCC 27592, complete genome	Sedimenticola sp. SIP-G1, complete genome
	Sphingobium sp. TKS chromosome 1, complete sequence	Shigella flexneri 1a strain 0228, complete genome	Salmonella enterica subsp. enterica serovar Typhimurium strain SO2, complete genome	Serratia liquefaciens strain FDAARGOS_125, complete genome	Spirochaeta thermophila DSM 6578, complete genome
	Spirometra erinaceieuropaei genome assembly S_erinaceieuropaei, scaffold SPER_scaffold0106679	Shigella flexneri G1663, complete genome	Salmonella enterica subsp. enterica serovar Typhimurium strain SO3, complete genome	Serratia liquefaciens strain HUMV-21, complete genome	Spirometra erinaceieuropaei genome assembly S_erinaceieuropaei, scaffold SPER_contig0037806
	Streptomyces griseus gene for non-ribosomal peptide synthetase, partial cds, strain: NBRC 15391, clone: N5	Shigella sonnei strain FDAARGOS_90, complete genome	Salmonella enterica subsp. enterica serovar Typhimurium strain YU15, complete genome	Solanum lycopersicum chromosome ch06, complete genome	Stenotrophomonas maltophilia D457 complete genome
	Streptomyces iranensis genome assembly Siranensis, scaffold SCAF00001	Shigella sonnei strain FORC_011, complete genome	Singulisphaera acidiphila DSM 18658, complete genome	Sphaeroforma arctica JP610 hypothetical protein partial mRNA	Streptomyces venezuelae genome assembly Streptomyces venezuelae ATCC 15439, chromosome : I
	Streptomyces rapamycinicus NRRL 5491 genome	Spirometra erinaceieuropaei genome assembly S_erinaceieuropaei, scaffold SPER_scaffold0001956	Sinorhizobium medicae WSM419, complete genome	Spirometra erinaceieuropaei genome assembly S_erinaceieuropaei, scaffold SPER_scaffold0054014	Streptomyces venezuelae strain ATCC 15439, complete genome
	Streptomyces reticuli genome assembly TUE45, chromosome : I	Staphylococcus epidermidis strain NW32 genomic sequence	Sodalis praecaptivus strain HS1, complete genome	Spirometra erinaceieuropaei genome assembly S_erinaceieuropaei, scaffold SPER_scaffold0086910	Syphacia muris genome assembly S_muris_Valencia, scaffold SMUV_scaffold0000363
	Streptomyces sp. CFMR 7 strain CFMR-7, complete genome	Stenotrophomonas acidaminiphila strain ZAC14D2_NAIMI4_2, complete genome	Streptomyces albus strain DSM 41398, complete genome	Spirometra erinaceieuropaei genome assembly S_erinaceieuropaei, scaffold SPER_scaffold0087230	
		Synechococcus sp. PCC 6312, complete genome	Streptomyces albus subsp. albus salinomycin biosynthesis cluster, strain DSM 41398	Spirometra erinaceieuropaei genome assembly S_erinaceieuropaei, scaffold SPER_scaffold0173320	
Taenia asiatica genome assembly T_asiatica_South_Korea, scaffold TASK_scaffold0000007	Thalassospira xiamenensis M-5 = DSM 17429, complete genome	Toxocara canis genome assembly T_canis_Equador, scaffold TCNE_scaffold0000352	Triticum aestivum chromosome 3B, genomic scaffold, cultivar Chinese Spring		Thecamonas trahens ATCC 50062 carboxylesterase type B partial mRNA

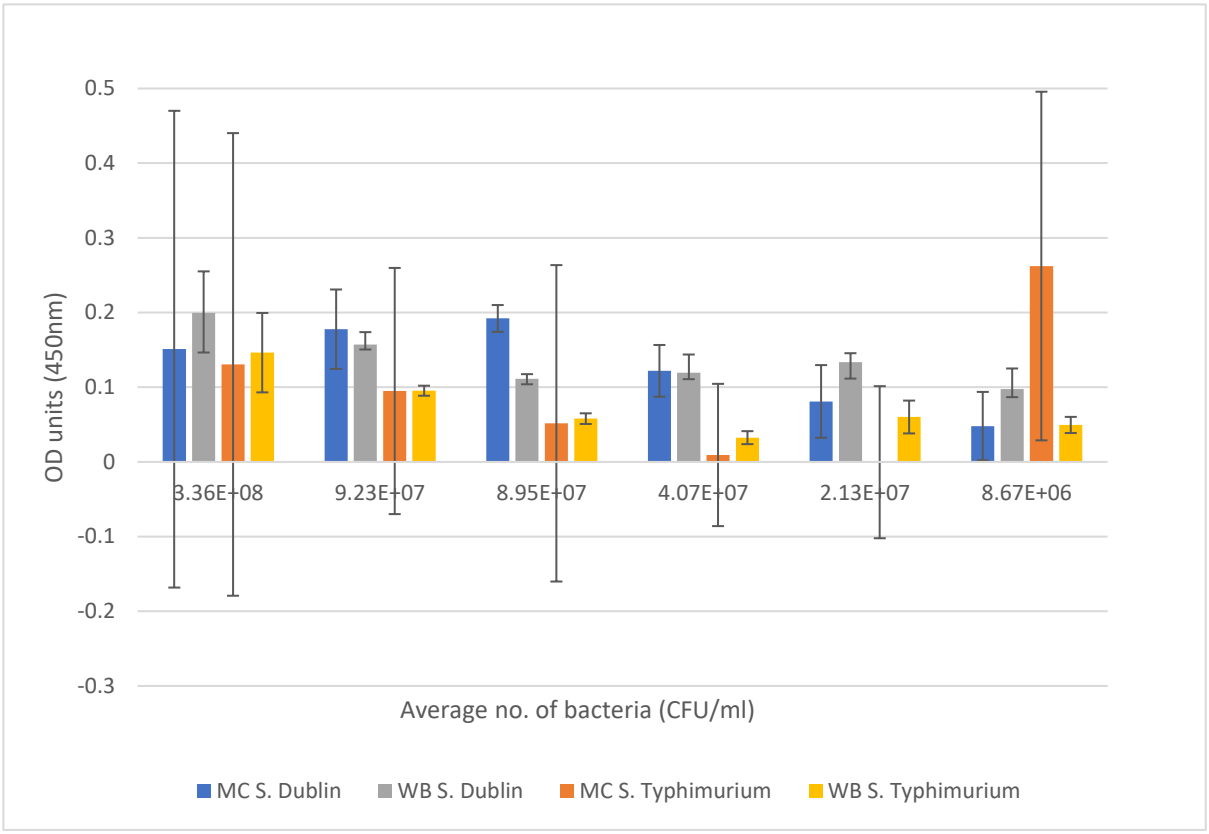
F3E	FIPE	FLoopE	BLoopE	BIPE	B3E
Thermotoga maritima MSB8, complete genome	Thecamonas trahens ATCC 50062 hypothetical protein partial mRNA	Toxocara canis genome assembly T_canis_Equador, scaffold TCNE_scaffold0001640			TPA: Oryzias latipes strain Hd-rR, complete genome assembly, chromosome 18
Thermotoga maritima MSB8, complete genome	Trypanosoma grayi structural maintenance of chromosome (SMC) family protein partial mRNA	Trichoderma atroviride IMI 206040 Hypothetical protein partial mRNA			Trichophyton rubrum CBS 118892 golgi transporter Sly1 (TERG_03821) mRNA, complete cds
Thermotoga maritima MSB8, complete genome					Trichophyton verrucosum HKI 0517 hypothetical protein, mRNA
Thermotoga maritima MSB8, complete genome					
Thermotoga maritima strain Tma100, complete genome					
Thermotoga maritima strain Tma200, complete genome					
Thermotoga naphthophila RKU-10, complete genome					
Thermotoga neapolitana DSM 4359, complete genome					
Thermotoga petrophila RKU-1, complete genome					
Thermotoga sp. 2812B, complete genome					
Thermotoga sp. Cell2, complete genome					
Thermotoga sp. RQ2, complete genome					
Thermotoga sp. RQ7, complete genome					
TPA: Neospora caninum Liverpool, chromosome chrII, complete genome					
		Uncultured virus clone contig ss9000001 genomic sequence	Uncultured bacterium 1114 genomic sequence	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: TSBAR002_C20	
			Uncultured bacterium partial 16S rRNA gene, clone OTU1290	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: TSBAR003_O08	
			Uncultured Jannaschia sp. clone GN8LFNR02HMHXD genomic sequence	Ustilago maydis 521 hypothetical protein partial mRNA	
		Vibrio fluvialis strain ATCC 33809 chromosome 2, complete sequence		Variovorax paradoxus B4 chromosome 1, complete sequence	
		Vibrio tritonius DNA, chromosome 1, complete genome, strain: JCM 16456		Variovorax paradoxus EPS, complete genome	
Xanthophyllomyces dendrorhous genome assembly Xden1, scaffold Scaffold_33			Xanthomonas arboricola pv. juglandis strain Xaj 417 genome		
			Xanthomonas campestris strain 17, complete genome		
				Zebrafish DNA sequence from clone CH211-168F7 in linkage group 14, complete sequence	

Appendix 3: The genome, accession number and base-pair co-ordinates for gene sequences used to develop primers.

Genome	Accession No.	Gene	Genome co-ordinates (base pair number)
Salmonella enterica subsp. *enterica* serovar Typhimurium str. LT2	NC_003197.1	bapA	2827580 - 2839053
		hilA	3019856 - 3021517
		orgA	3014425 - 3015024

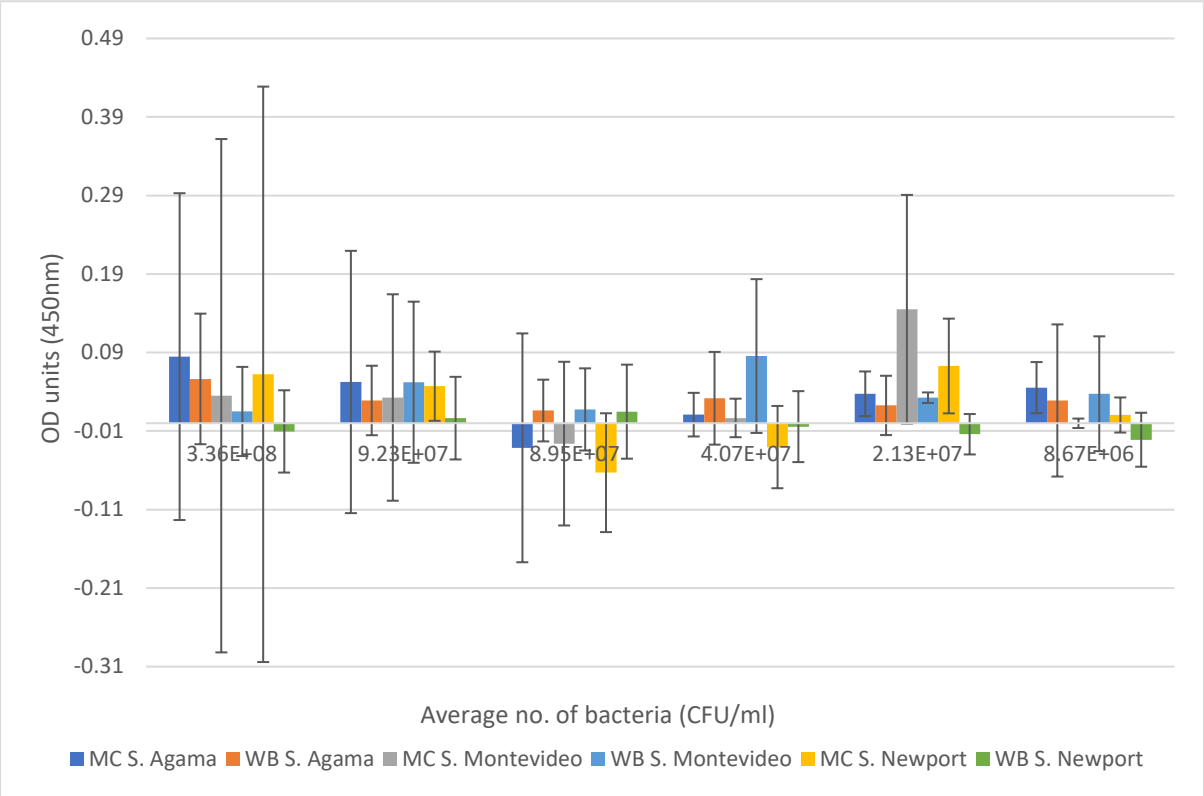
Appendix 4: How different wash steps affect the efficacy of the direct ELISA assay over a range of different bacterial concentrations when comparing *S.* Dublin and *S.* Typhimurium. The antigen step incubated at 37°C and the antibody used was monoclonal A99H. (n=3)

Key: MC = Multichannel wash steps, WB = Wash-bottle wash steps

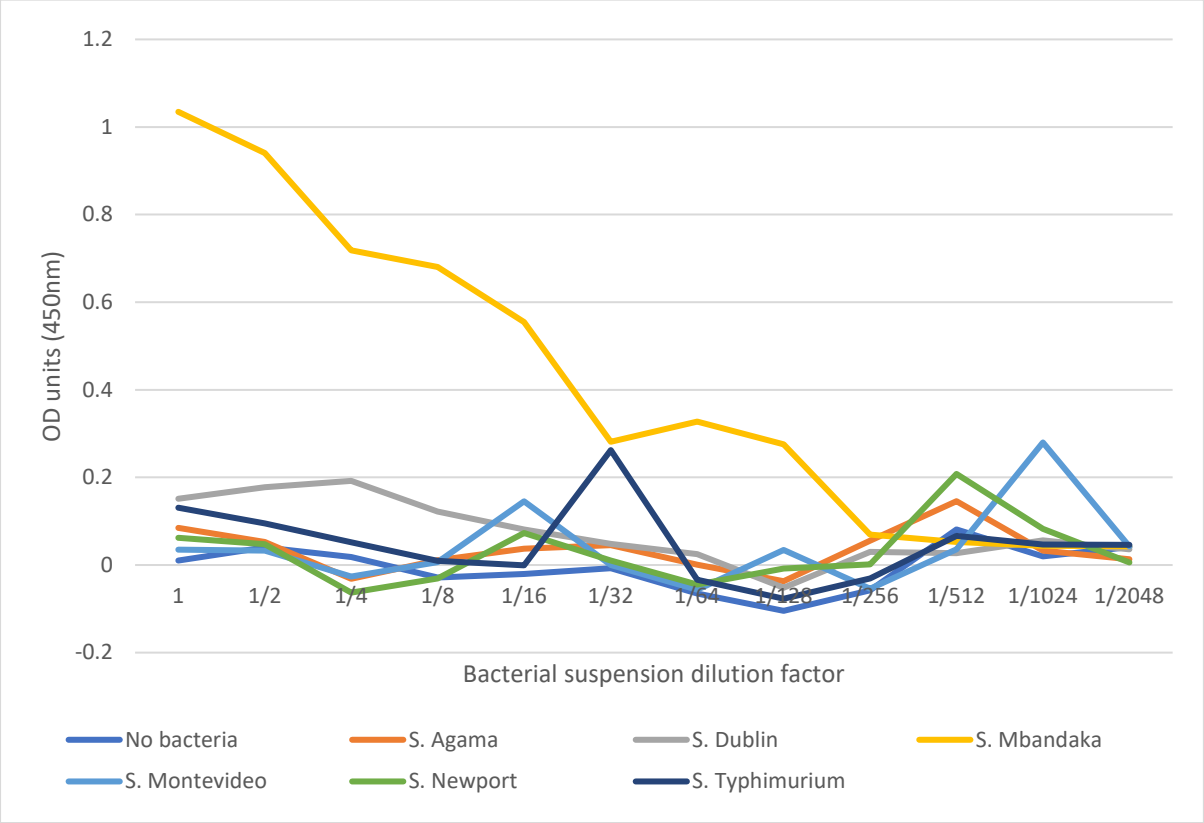


Appendix 5: How different wash steps affect the efficacy of the direct ELISA assay over a range of different bacterial concentrations when comparing *S.* Agama, *S.* Montevideo and *S.* Newport. The antigen step incubated at 37°C and the antibody used was monoclonal A99H. (n=3)

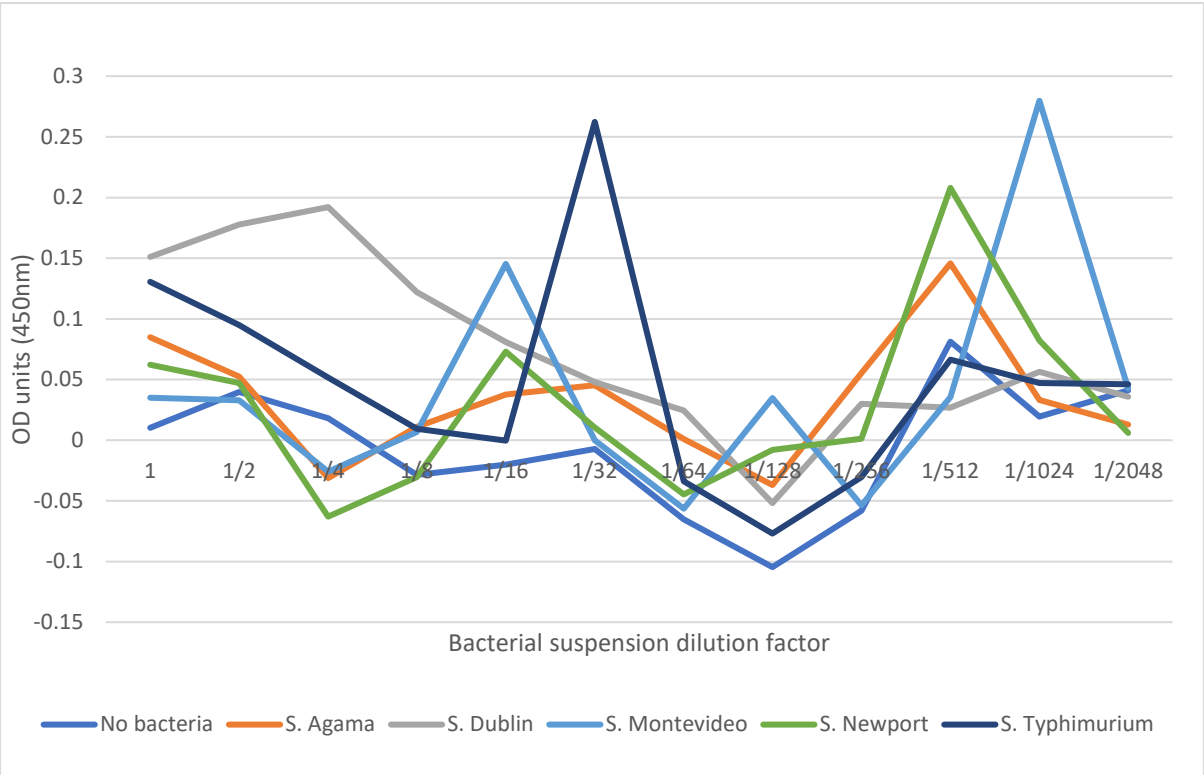
Key: MC = Multichannel wash steps, WB = Wash bottle wash steps



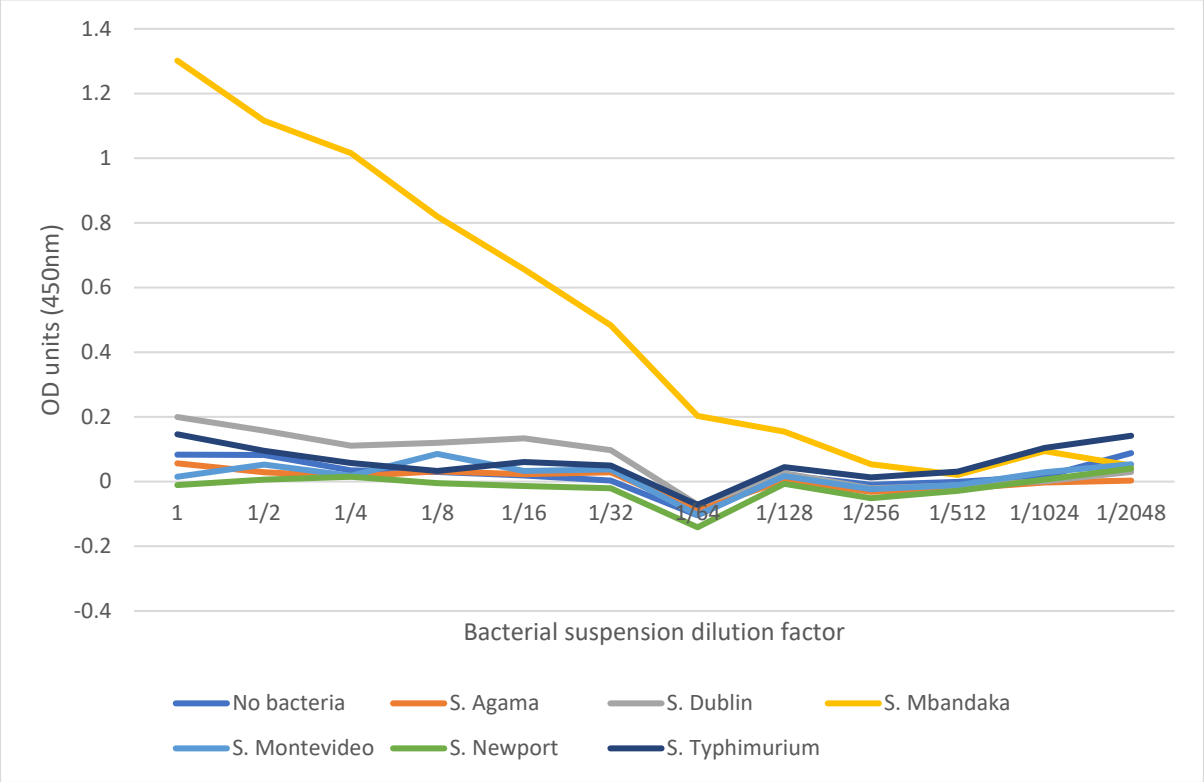
Appendix 6A: Detection of multiple *Salmonella* serovars over a range of different bacterial concentrations by monoclonal antibody A99H when incubated at 37°C, using a multichannel pipette to wash plates.



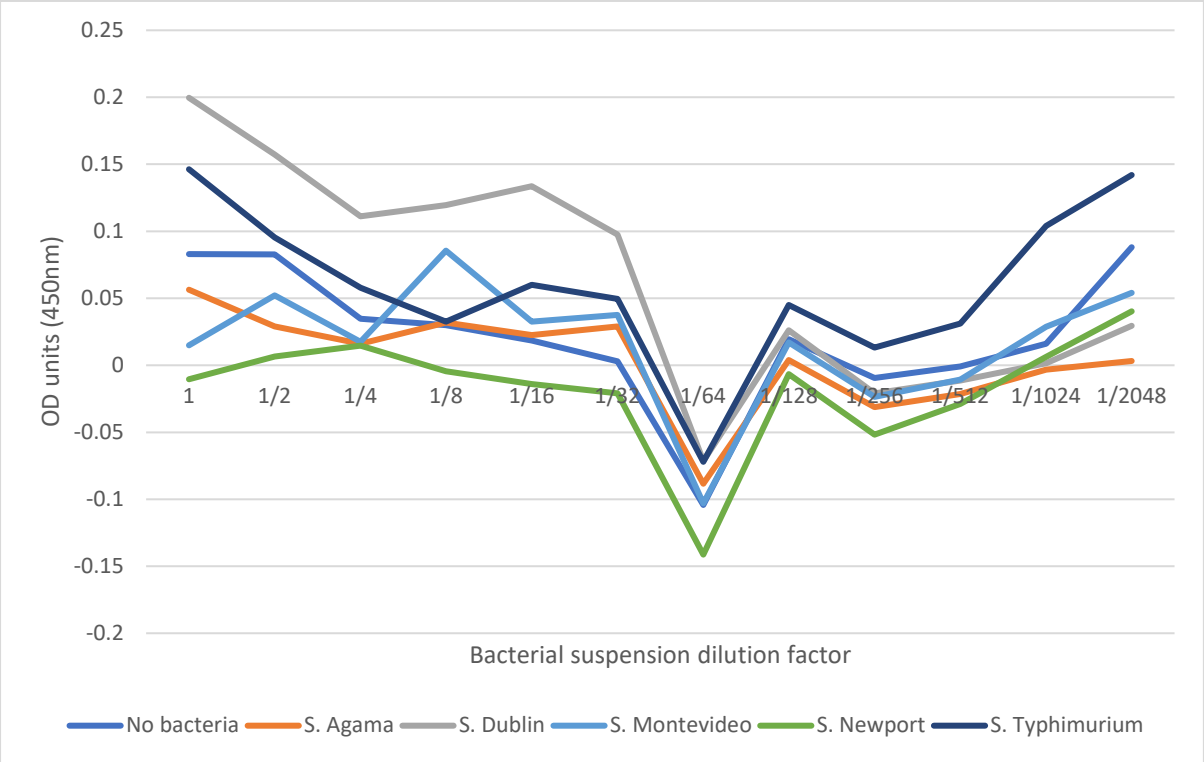
Appendix 6B: The data seen in appendix 6A, with *S.* Mbandaka removed. Detection of multiple *Salmonella* serovars over a range of different bacterial concentrations by monoclonal antibody A99H when incubated at 37°C, using a multichannel pipette to wash plate



Appendix 7A: Detection of multiple *Salmonella* serovars over a range of different bacterial concentrations by monoclonal antibody A99H when incubated at 37°C, using a wash bottle to wash plates.

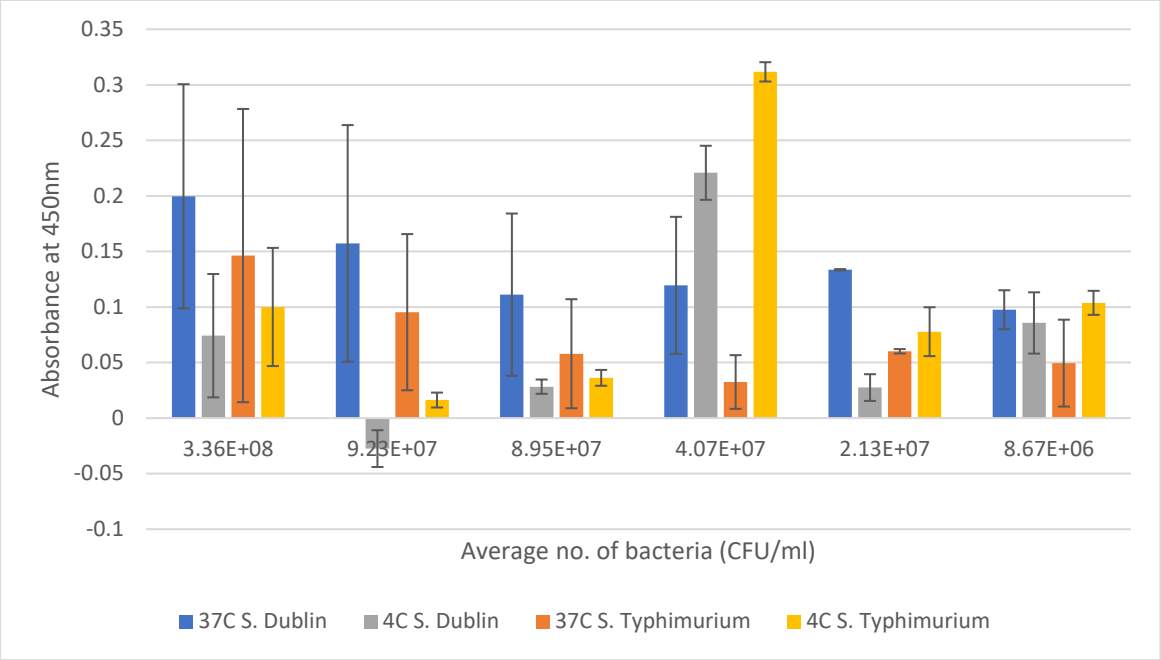


Appendix 7B: The data seen in appendix 7A, with *S.* Mbandaka removed. Detection of multiple *Salmonella* serovars over a range of different bacterial concentrations by monoclonal antibody A99H when incubated at 37°C, using a wash bottle to wash plates



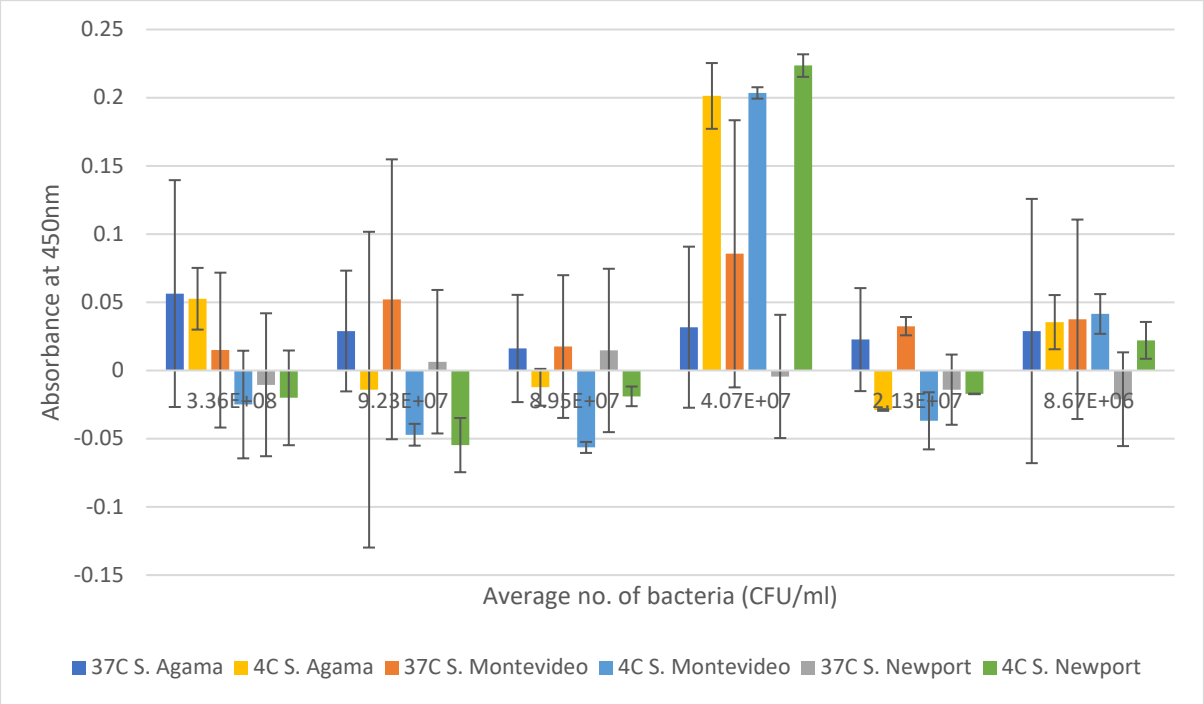
Appendix 8: How differing the temperature of the antigen incubation step in direct ELISA protocol affects the efficacy of *S.* Dublin and *S.* Typhimurium detection by monoclonal antibody A99H by over a range of different bacterial concentrations. WB wash steps were used. (n=3)

Key: 37C = antigen incubated at 37°C, 4C = antigen incubated at 4°C

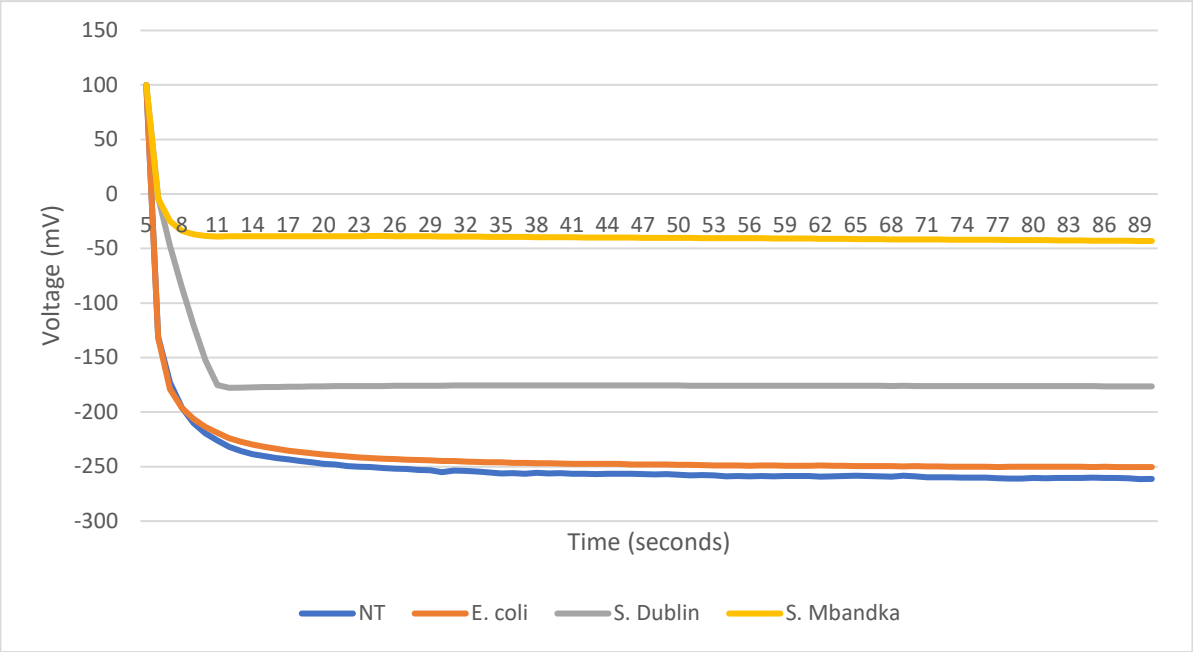


Appendix 9: How differing the temperature of the antigen incubation step in direct ELISA protocol affects the efficacy of *S.* Agama, *S.* Montevideo and *S.* Newport detection by monoclonal antibody A99H by over a range of different bacterial concentrations. WB wash steps were used. (n=3)

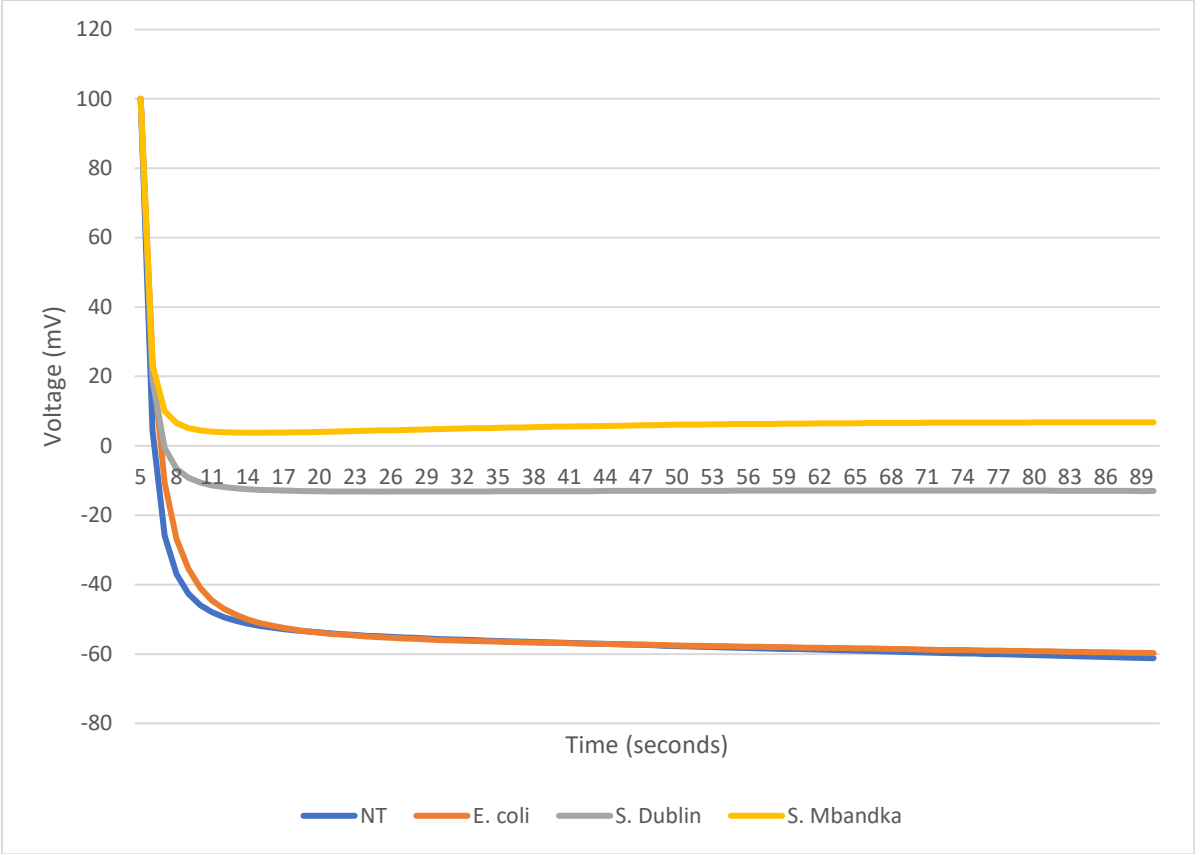
Key: 37C = antigen incubated at 37°C, 4C = antigen incubated at 4°C



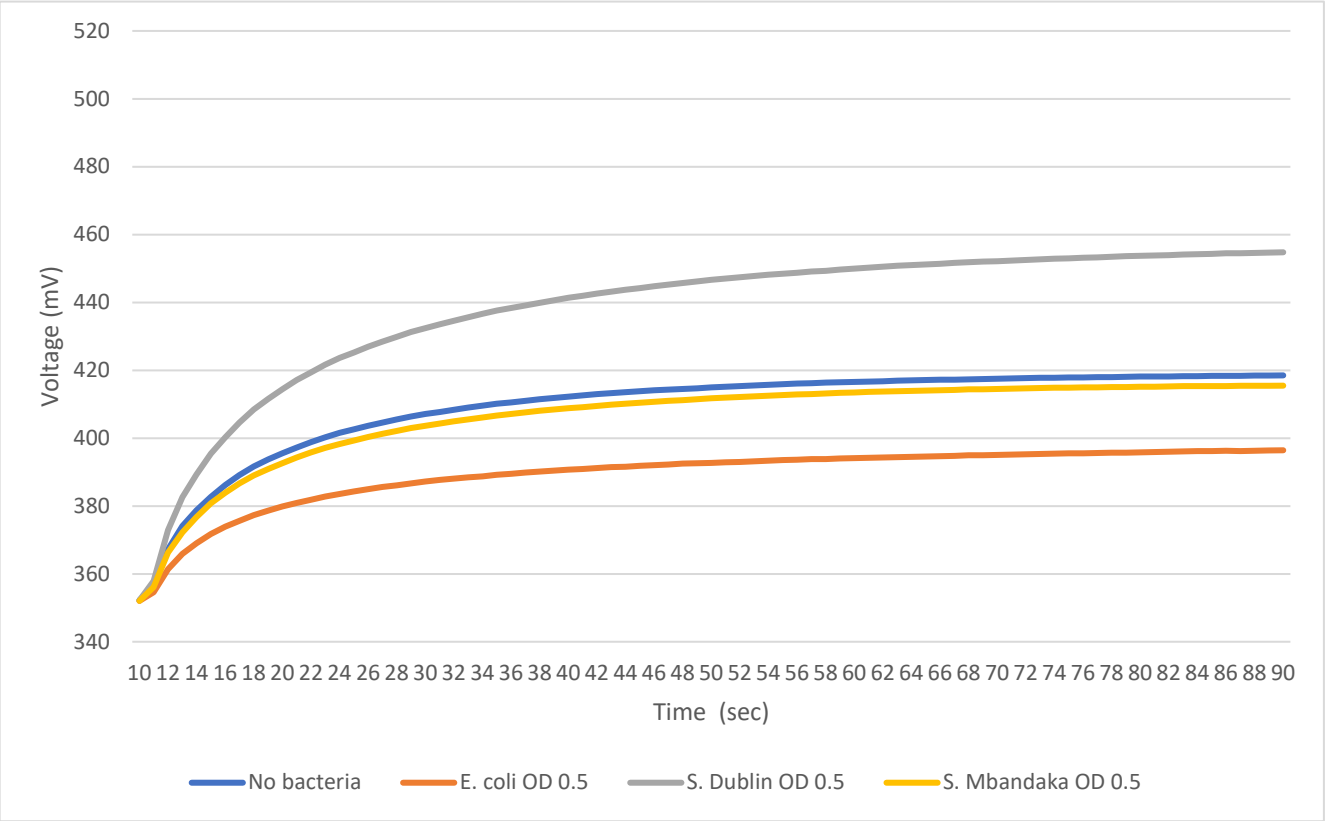
Appendix 10: Average of direct immunoassays on the VR1, with conjugated polyclonal TRP antibody incubated for 2 hours. Bacteria was standardised to 0.25 OD units (n=3)



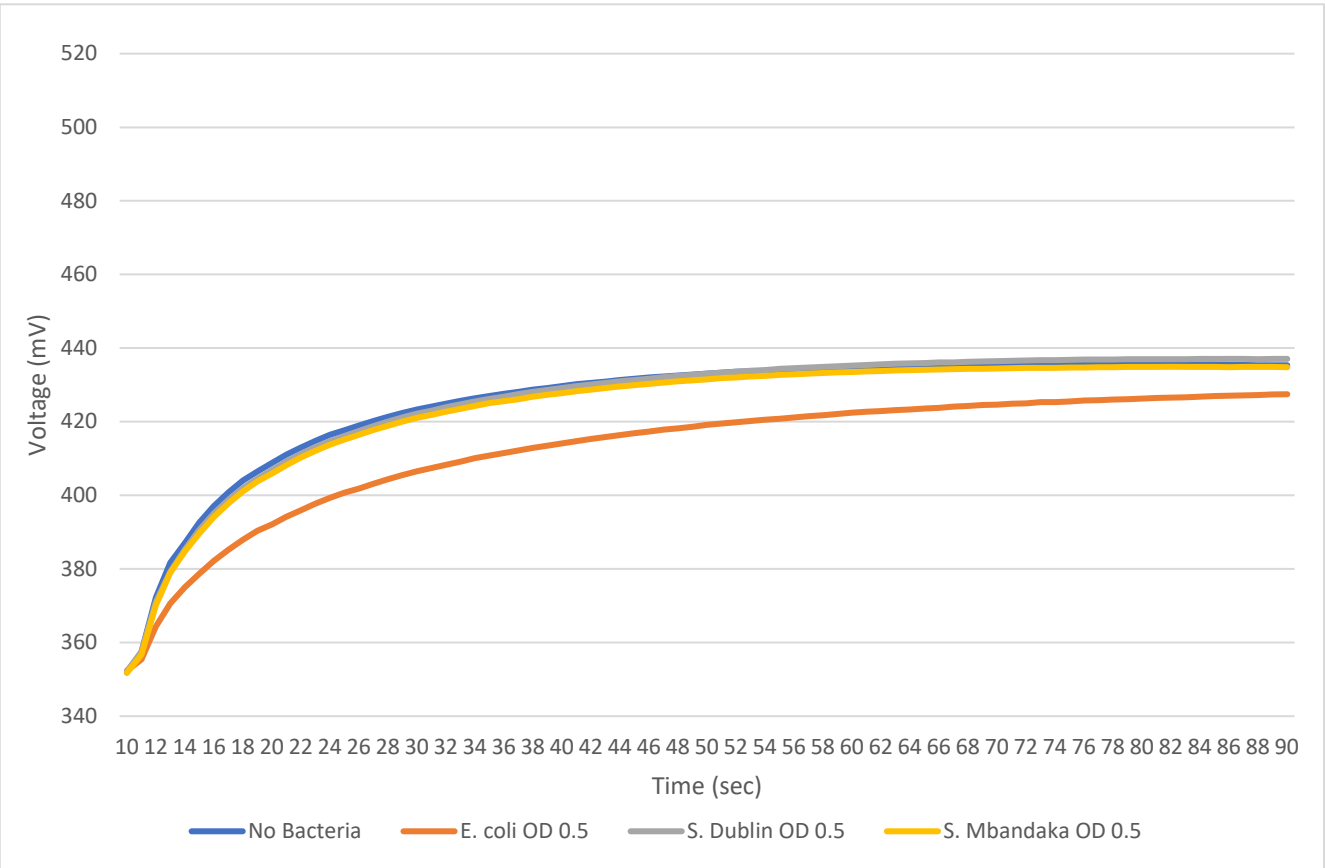
Appendix 11: Average of direct immunoassays on the VR1, with conjugated polyclonal TRP antibody incubated for 45 minutes. Bacteria was standardised to 0.25 OD units



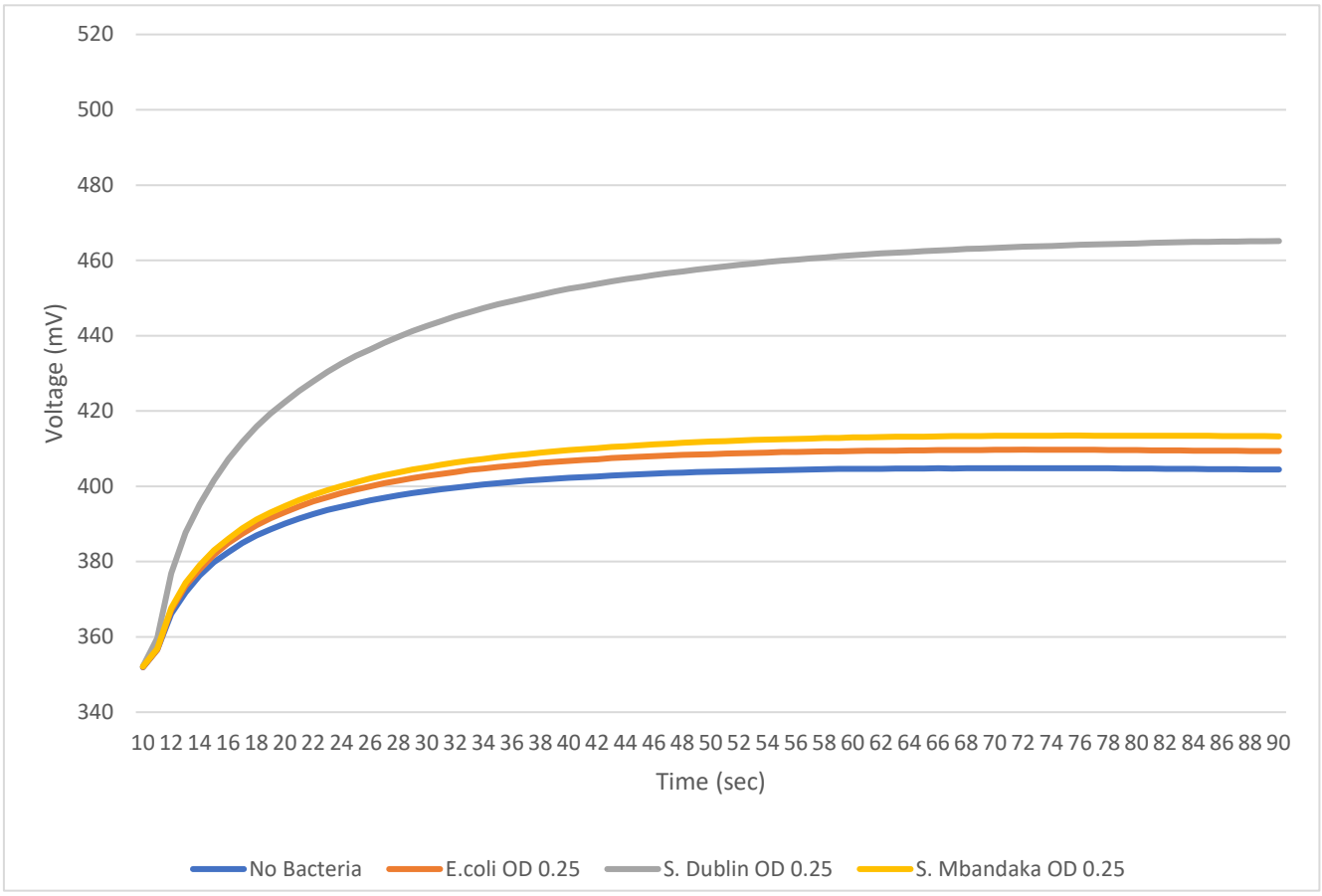
Appendix 12: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 2hr overall incubation time. Bacteria was standardised at 0.5 OD units.



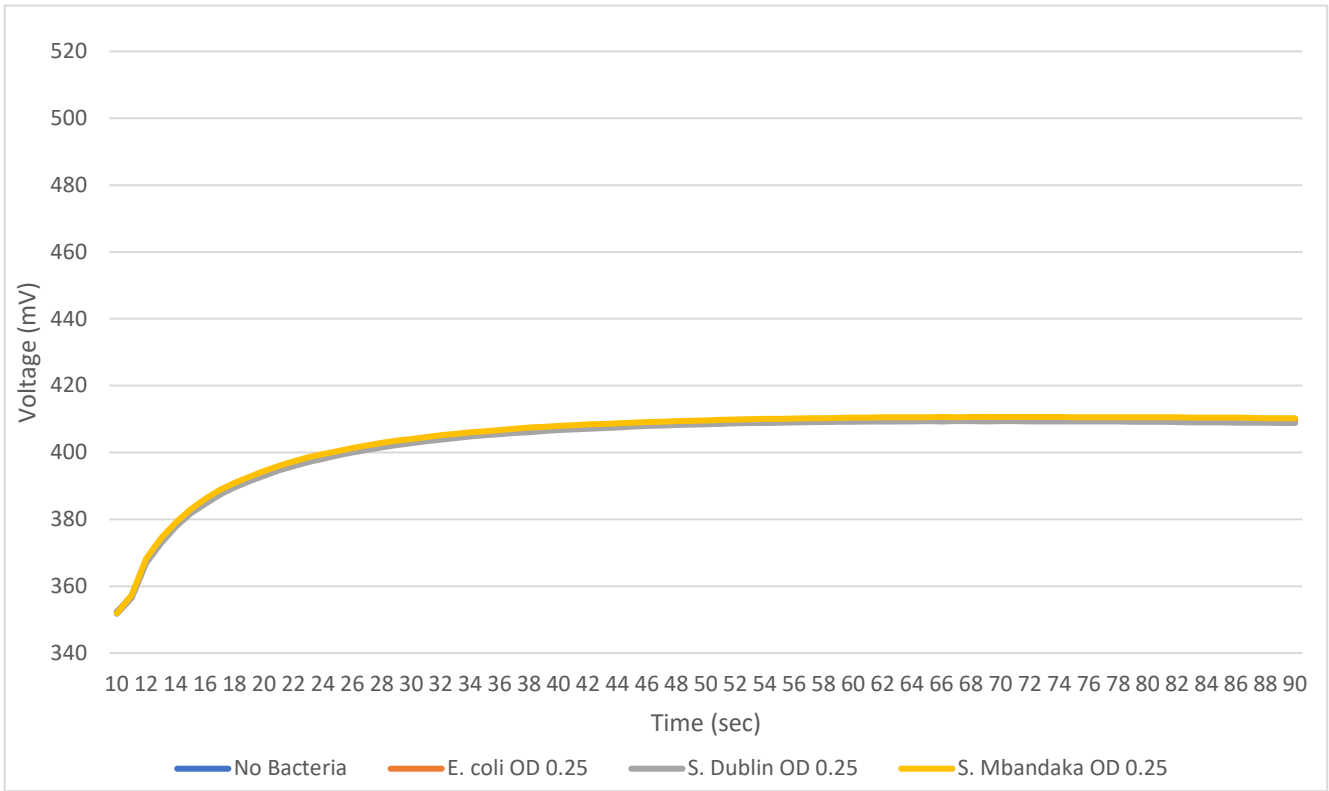
Appendix 13: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:500 dilution, using 2hr overall incubation time. Bacteria was standardised at 0.5 OD units.



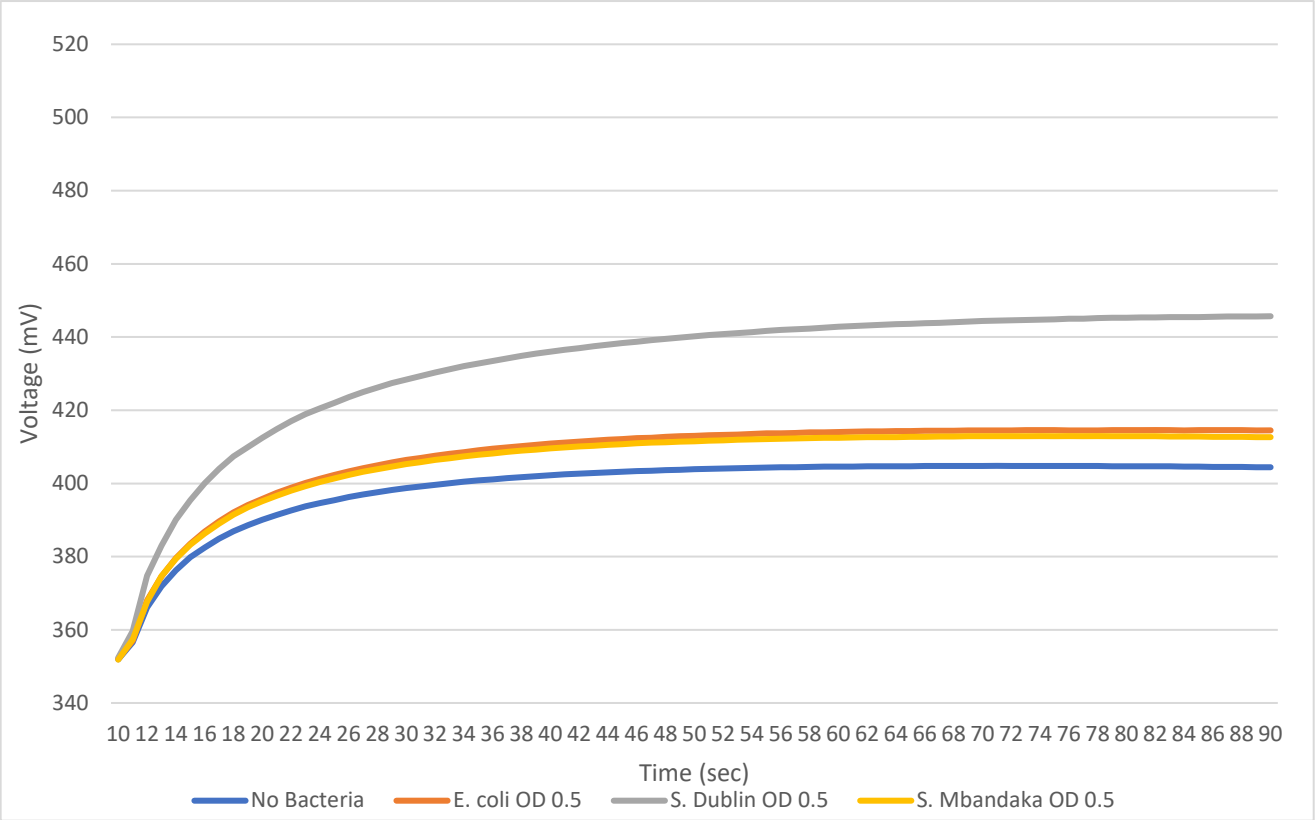
Appendix 14: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 2.5hr overall incubation time. Bacteria was standardised at 0.25 OD units.



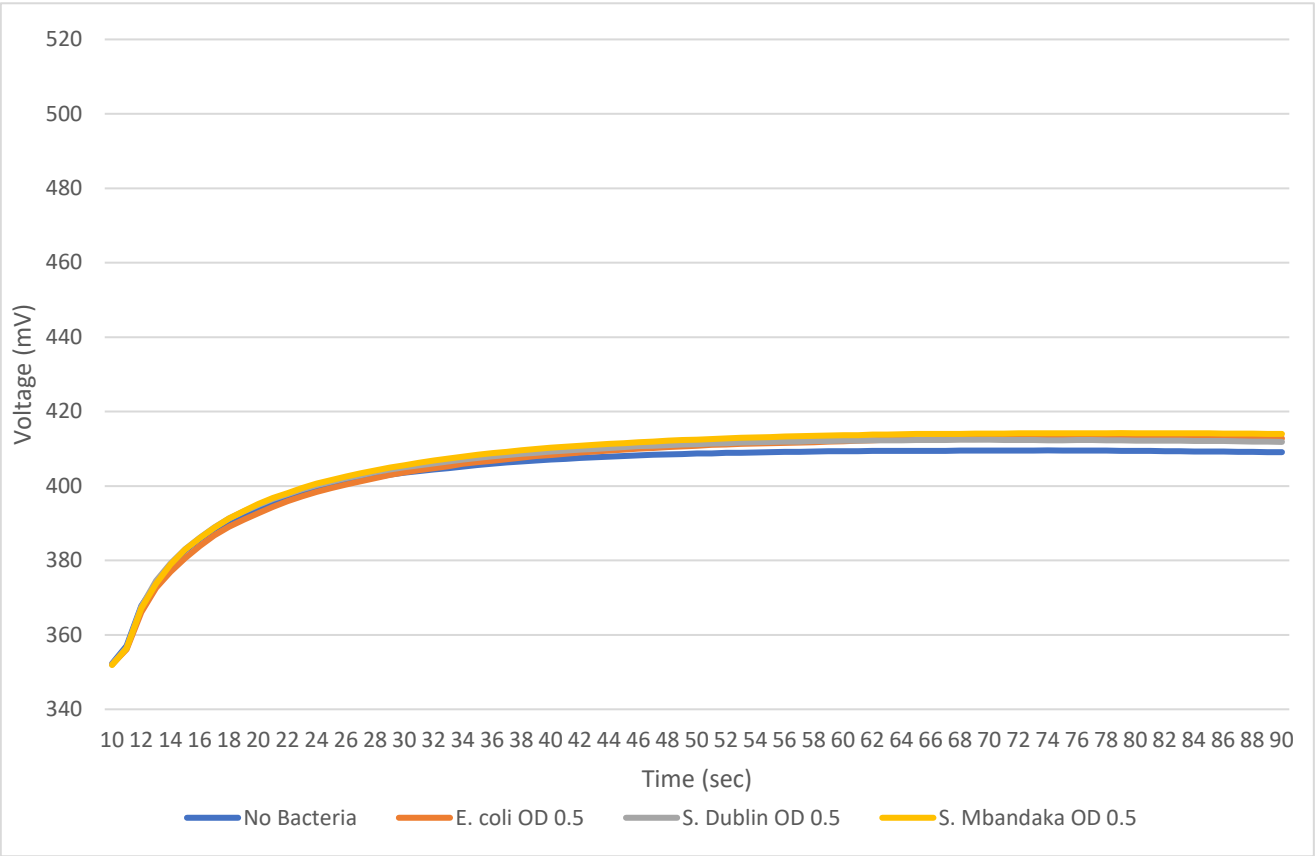
Appendix 15: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:500 dilution, using 2.5hr overall incubation time. Bacteria was standardised at 0.25 OD units.



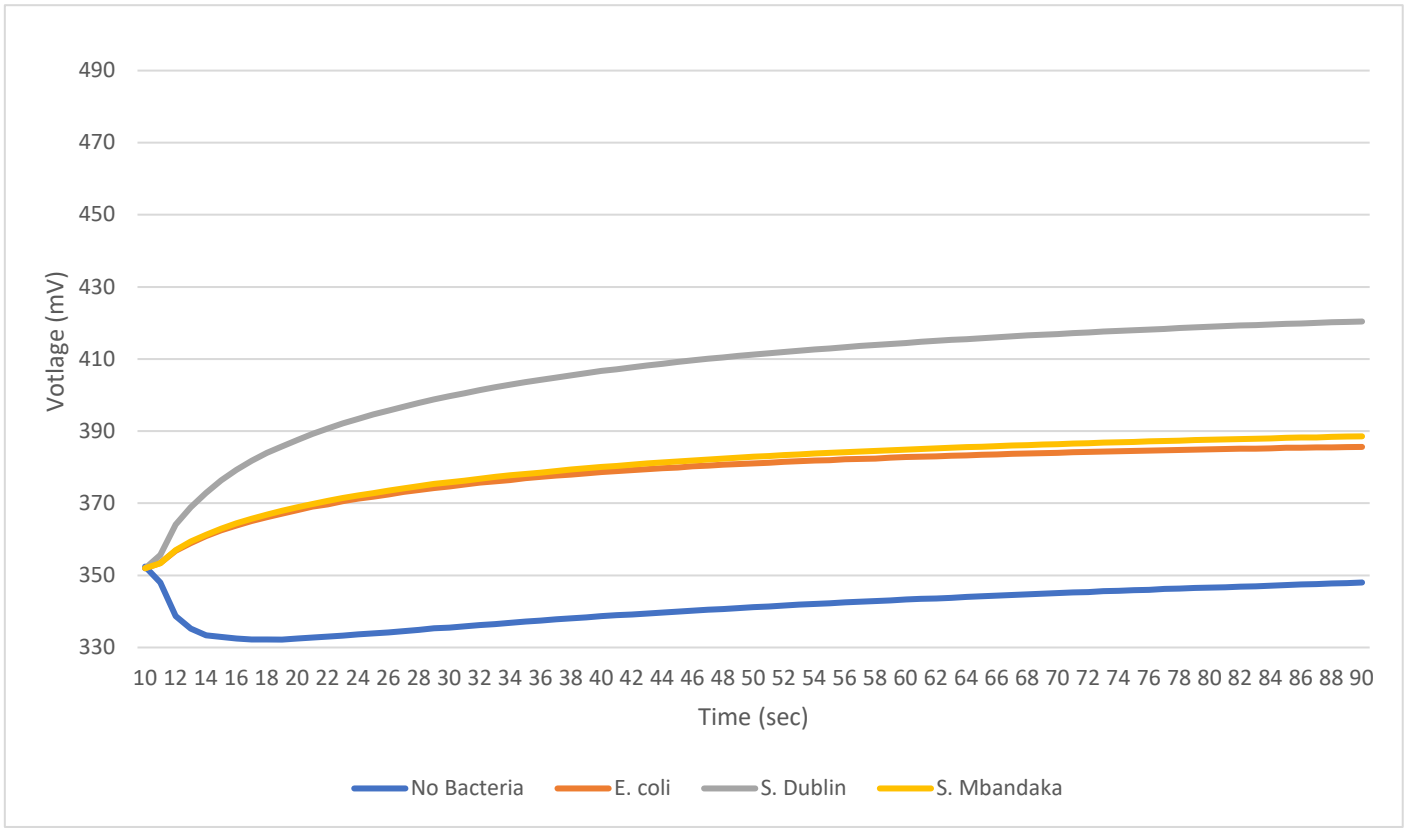
Appendix 16: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 2.5hr overall incubation time. Bacteria was standardised at 0.5 OD units



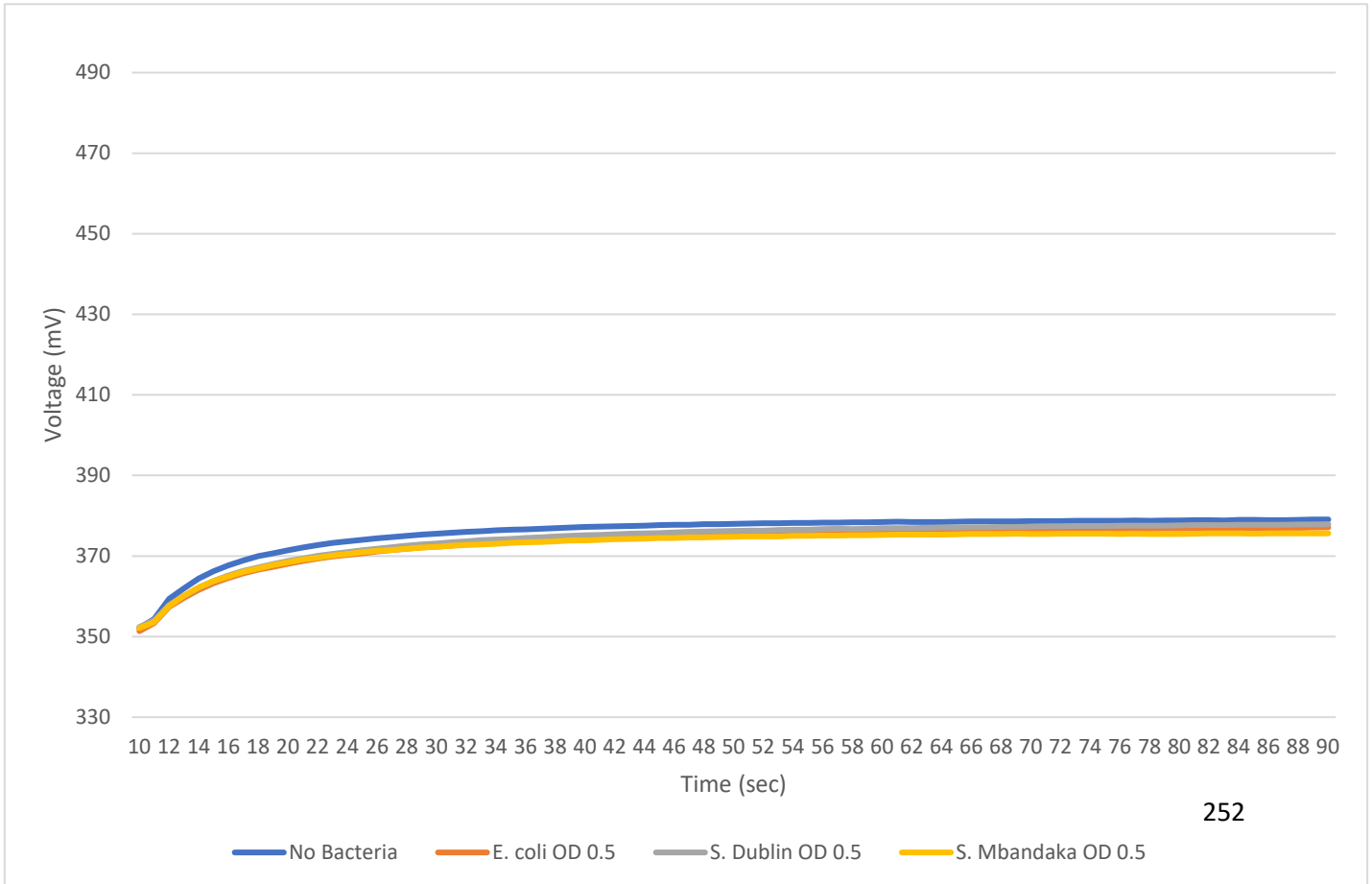
Appendix 17: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:500 dilution, using 2.5hr overall incubation time. Bacteria was standardised at 0.5 OD units



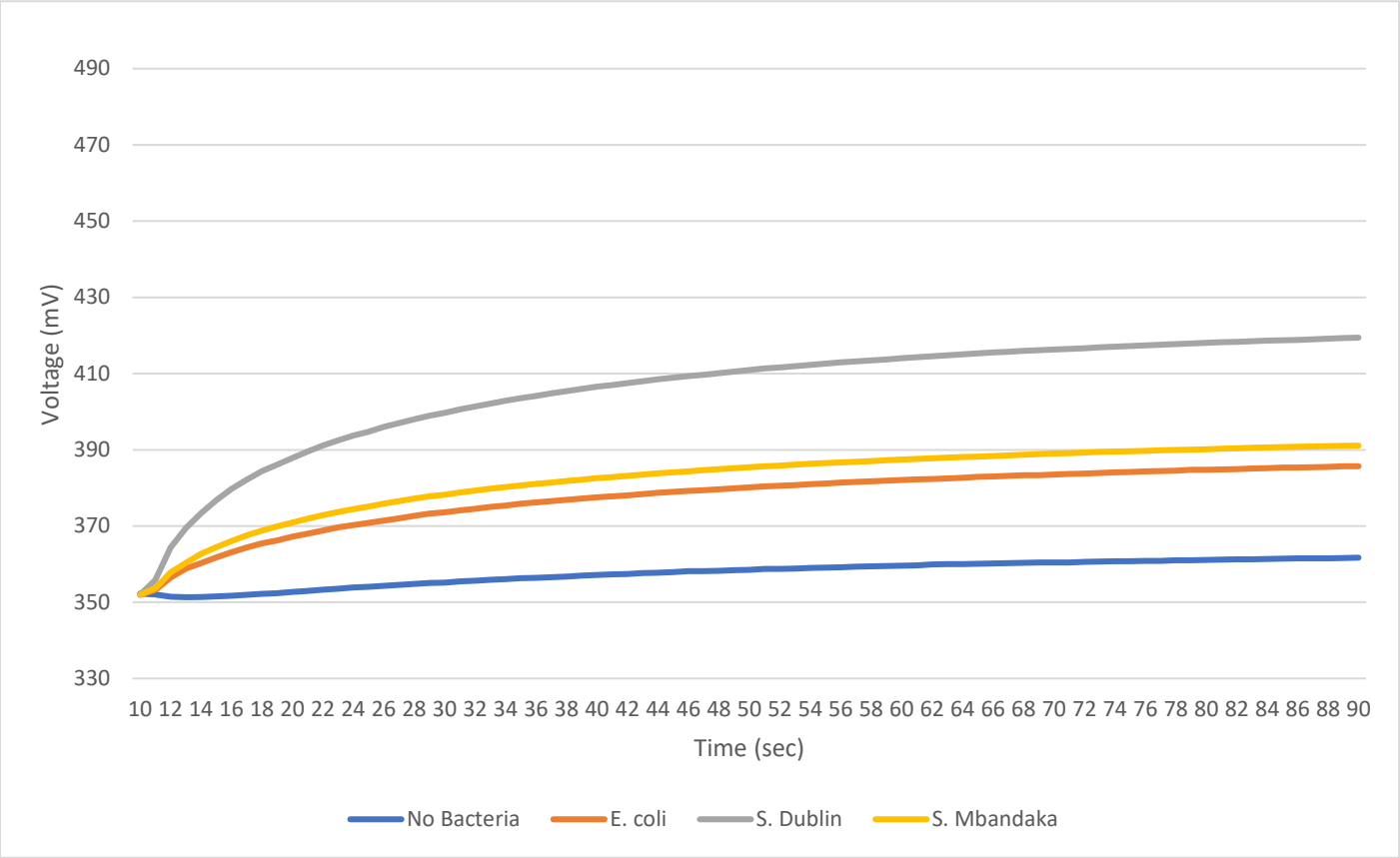
Appendix 18: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 1.75hr overall incubation time. Bacteria was standardised at 0.5 OD units



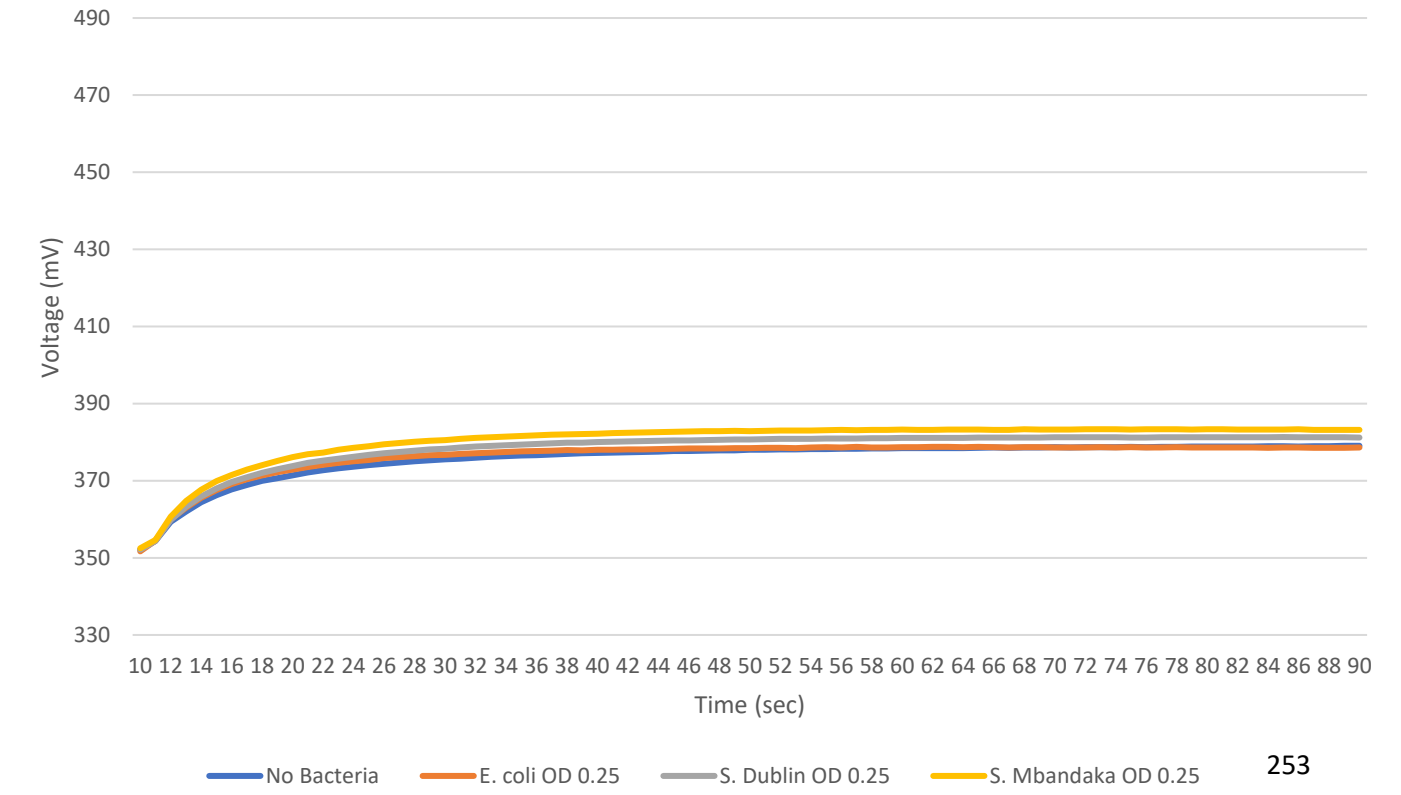
Appendix 19: Vantix Sandwich assay run on the VR2, with A99H at 1:100 dilution, using 1hr overall incubation time. Bacteria was standardised at 0.5 OD units



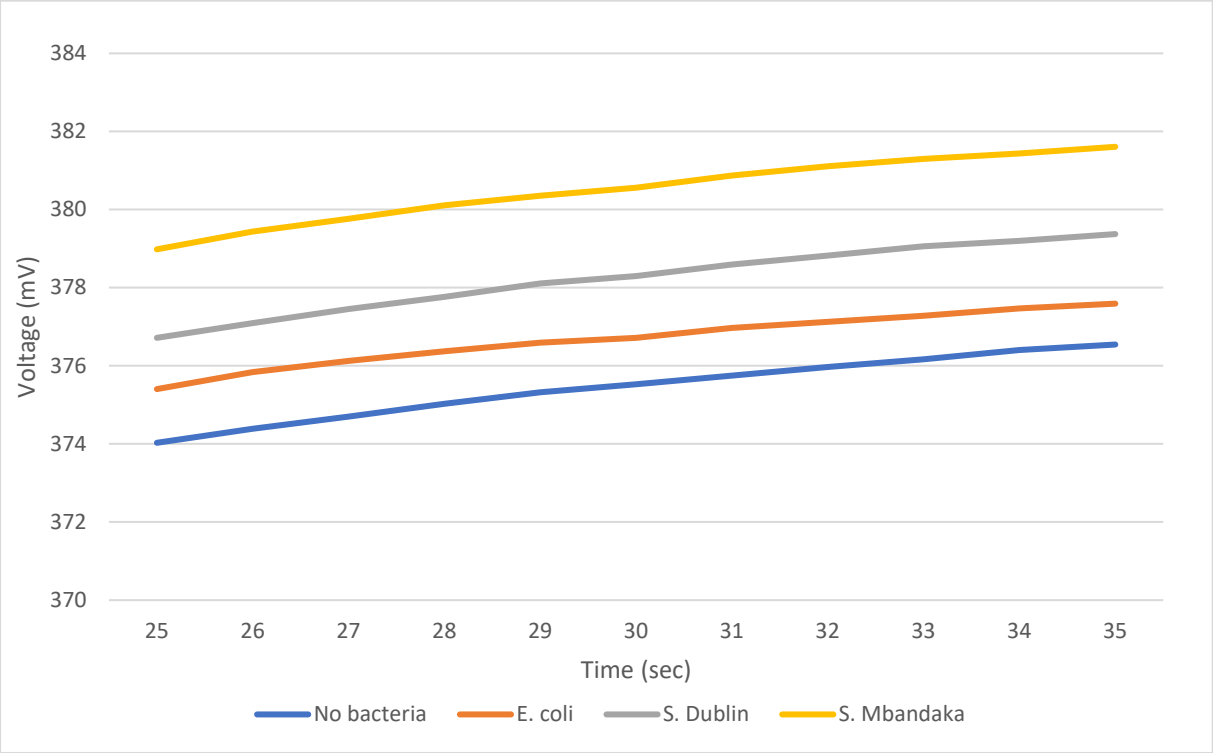
Appendix 20: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 1.75hr overall incubation time. Bacteria was standardised at 0.25 OD units.



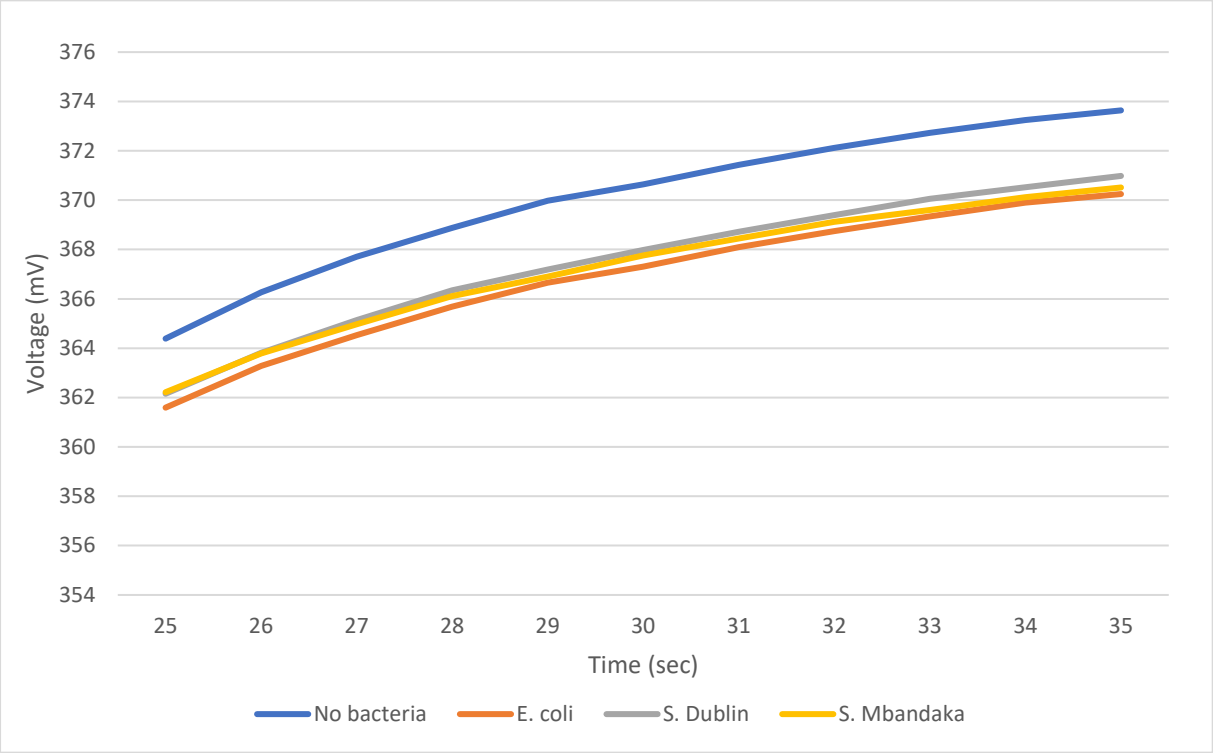
Appendix 21: Vantix Sandwich assay run on the VR2, with A99H at 1:100 dilution, using 1hr overall incubation time. Bacteria was standardised at 0.25 OD units.



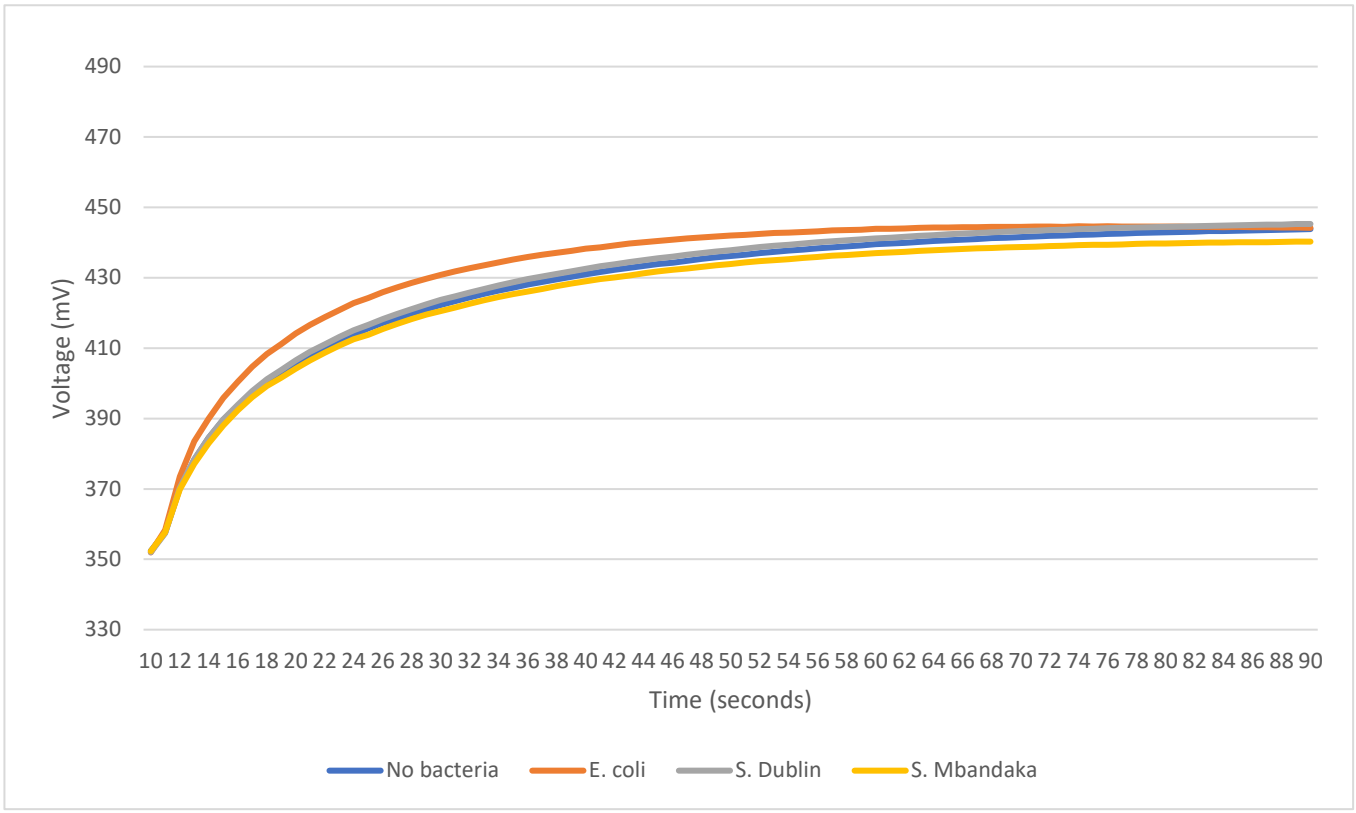
Appendix 22: A closer view of appendix 21, Vantix Sandwich assay run on the VR2, with A99H at 1:100 dilution, using 1hr overall incubation time, showing the 30sec timepoint. Bacteria was standardised at 0.25 OD units.



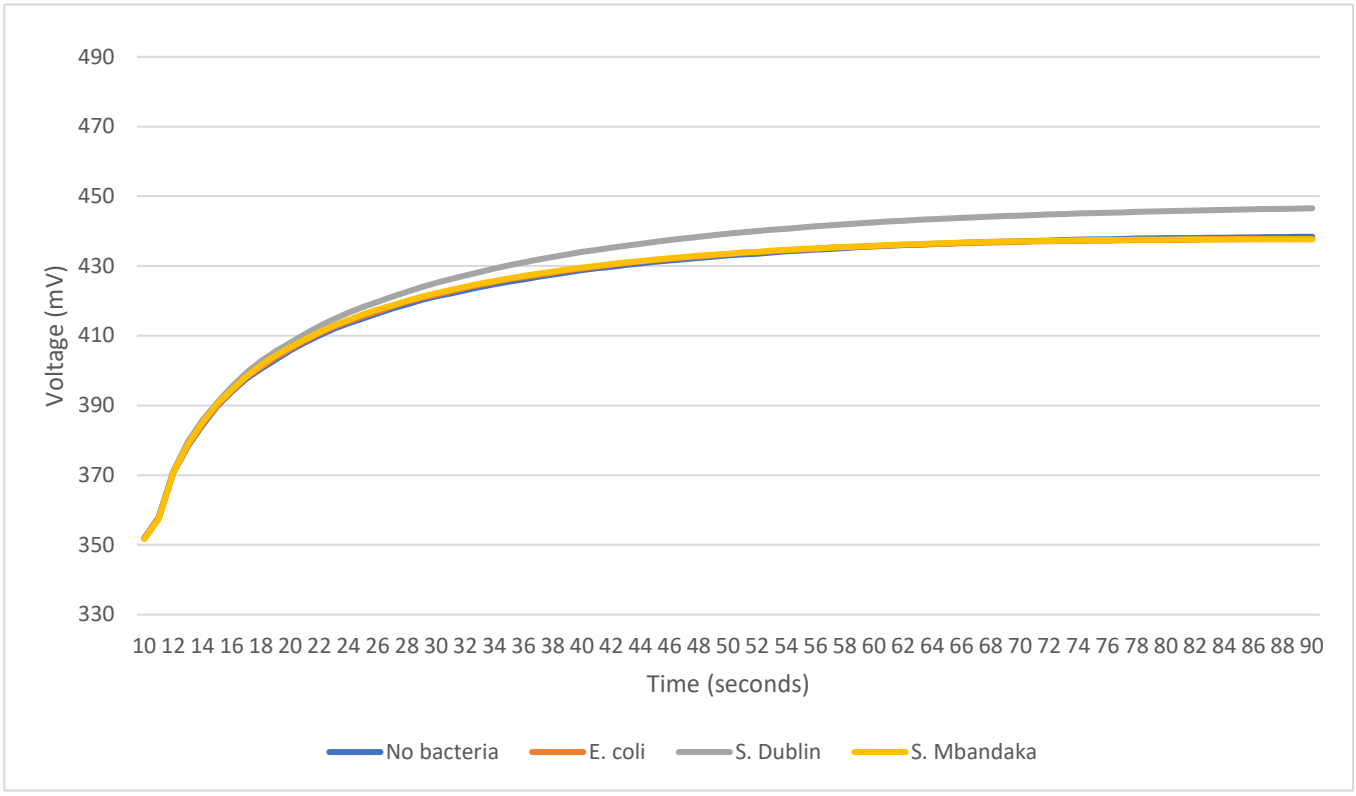
Appendix 23: A closer view of figure appendix 19, Vantix Sandwich assay run on the VR2, with A99H at 1:100 dilution, using 1hr overall incubation time, showing the 30sec timepoint. Bacteria was standardised at 0.5 OD units.



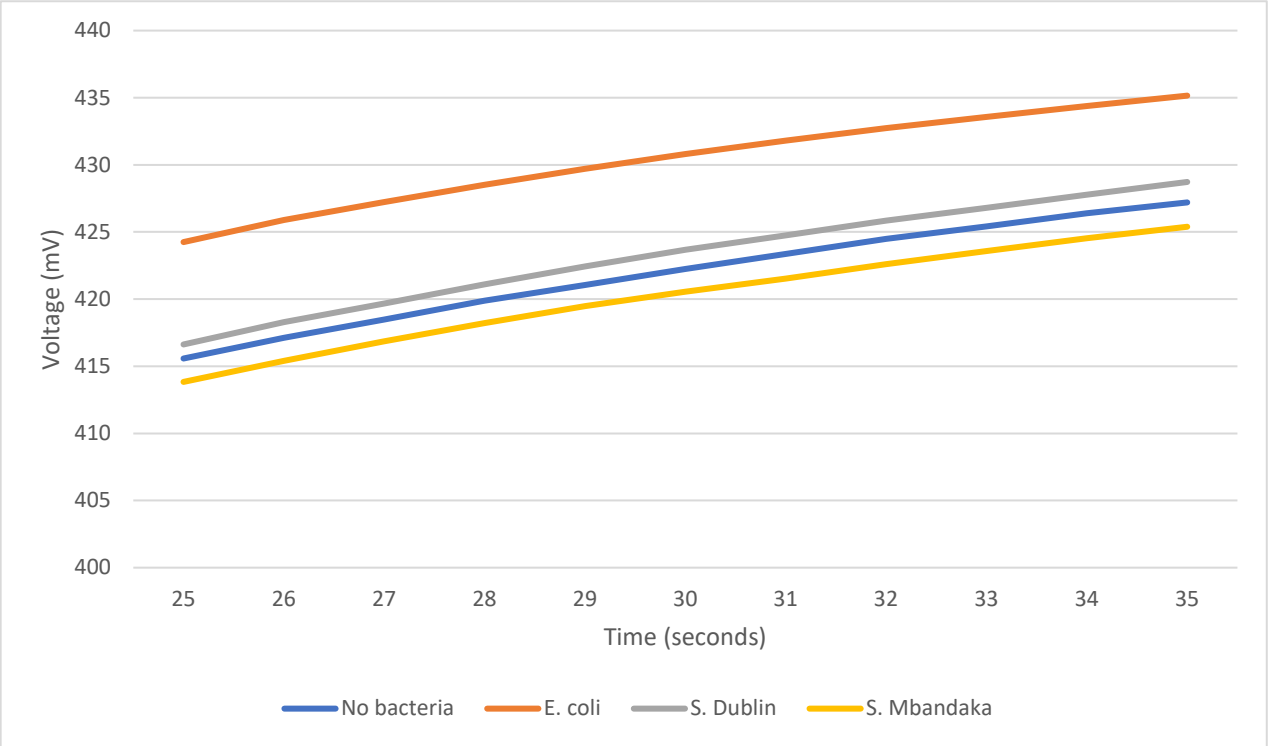
Appendix 24: Vantix Sandwich assay run on the VR2, with A99H at 1:100 dilution, using 40mins overall incubation time. Bacteria was standardised at 0.25 OD units.



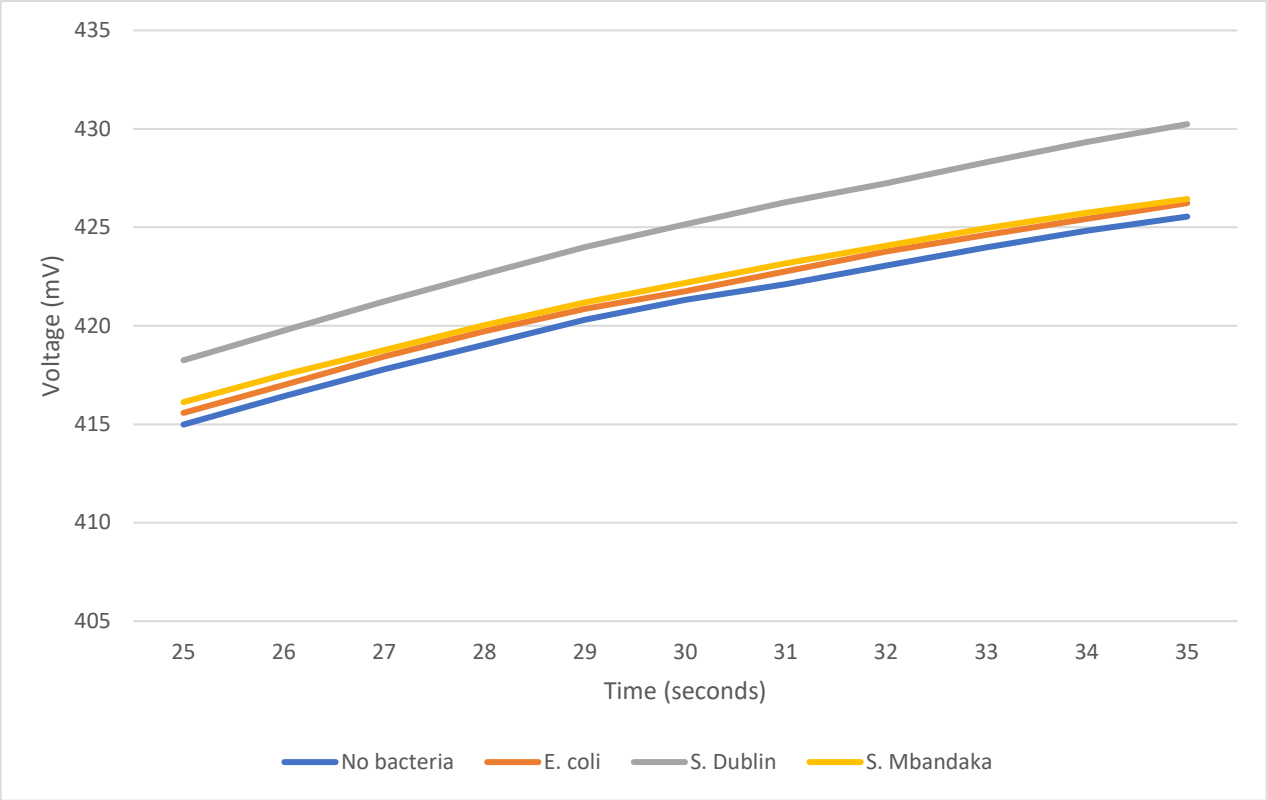
Appendix 25: Vantix Sandwich assay run on the VR2, with A99H at 1:100 dilution, using 40mins overall incubation time. Bacteria was standardised at 0.5 OD units.



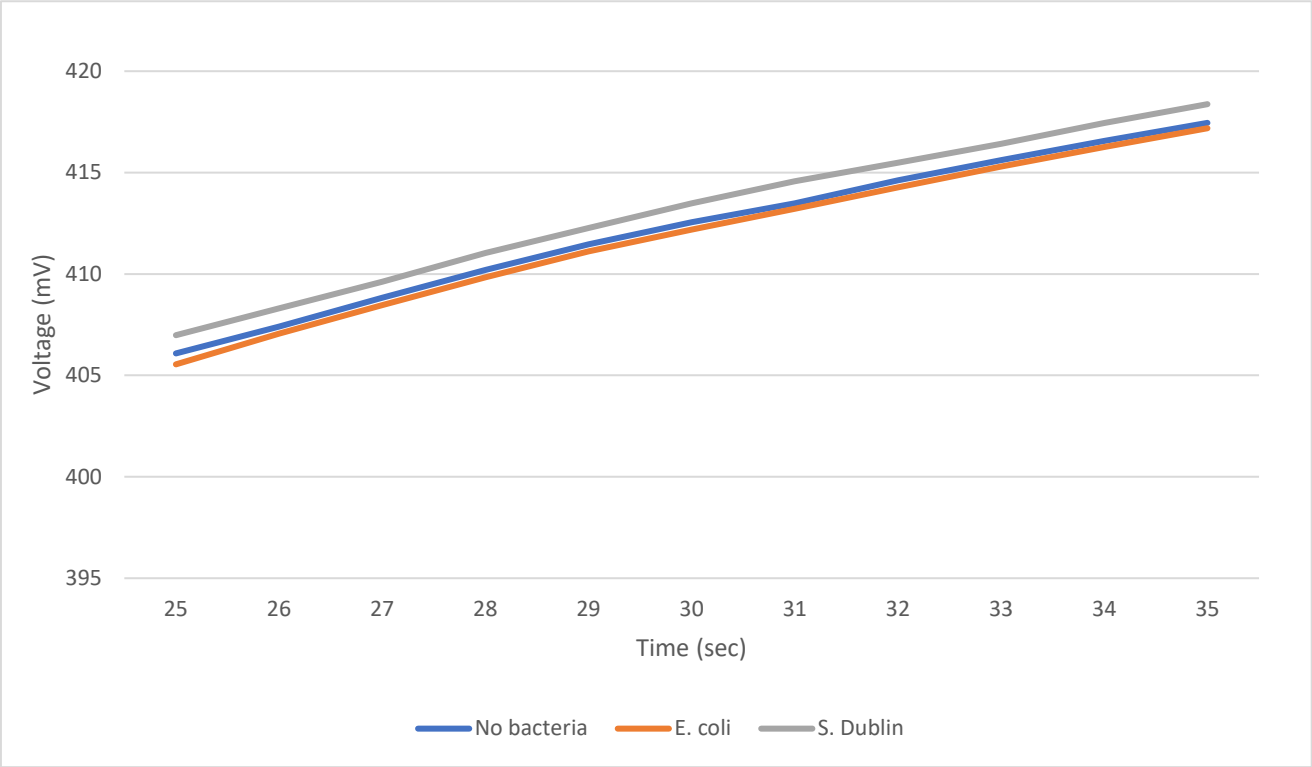
Appendix 26: A closer view of figure appendix 24, with A99H at 1:100 dilution, using 40 mins overall incubation time, showing the 30 sec timepoint. Bacteria was standardised at 0.25 OD units.



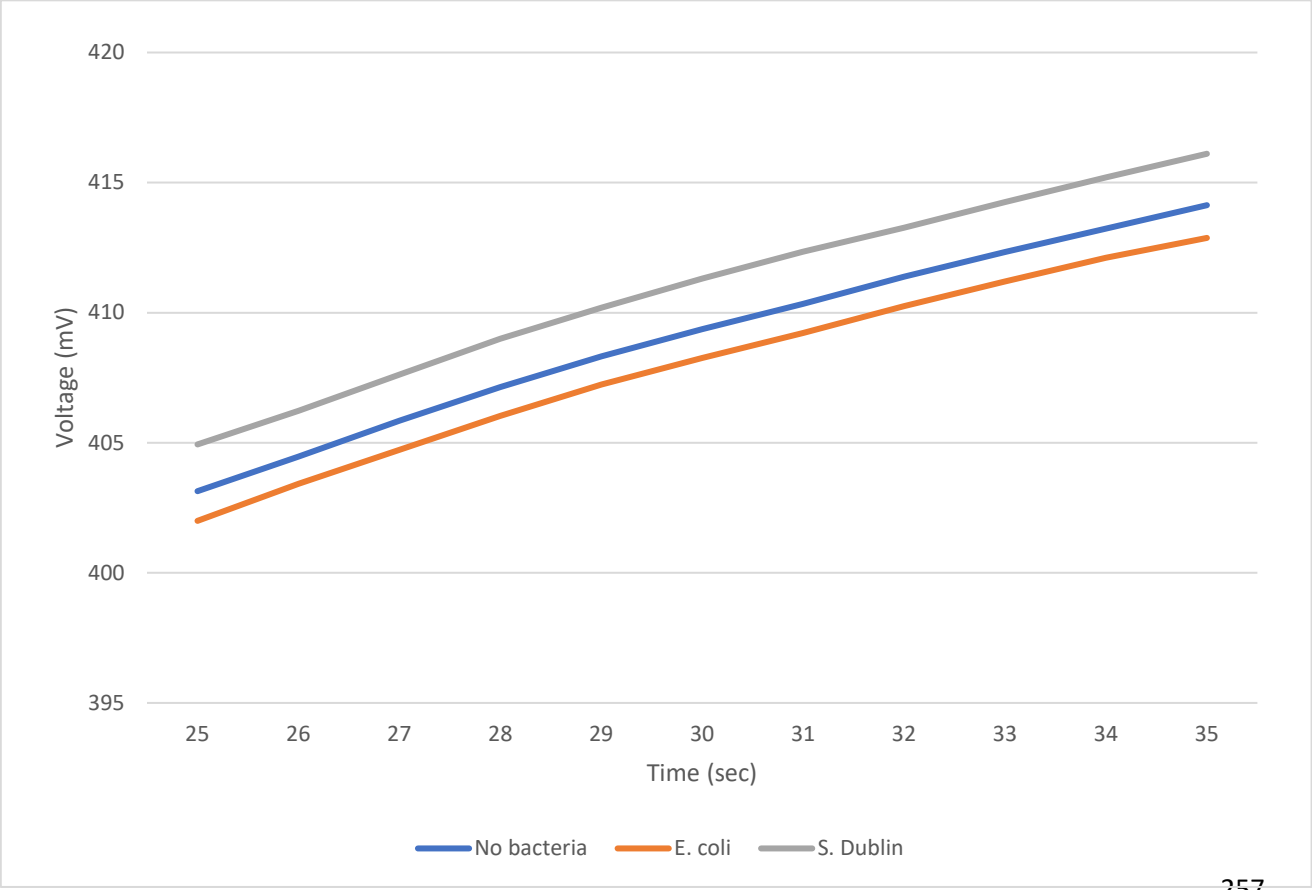
Appendix 27: A closer view of figure appendix 25, with A99H at 1:100 dilution, using 40 mins overall incubation time, showing the 30 sec timepoint. Bacteria was standardised at 0.5 OD units.



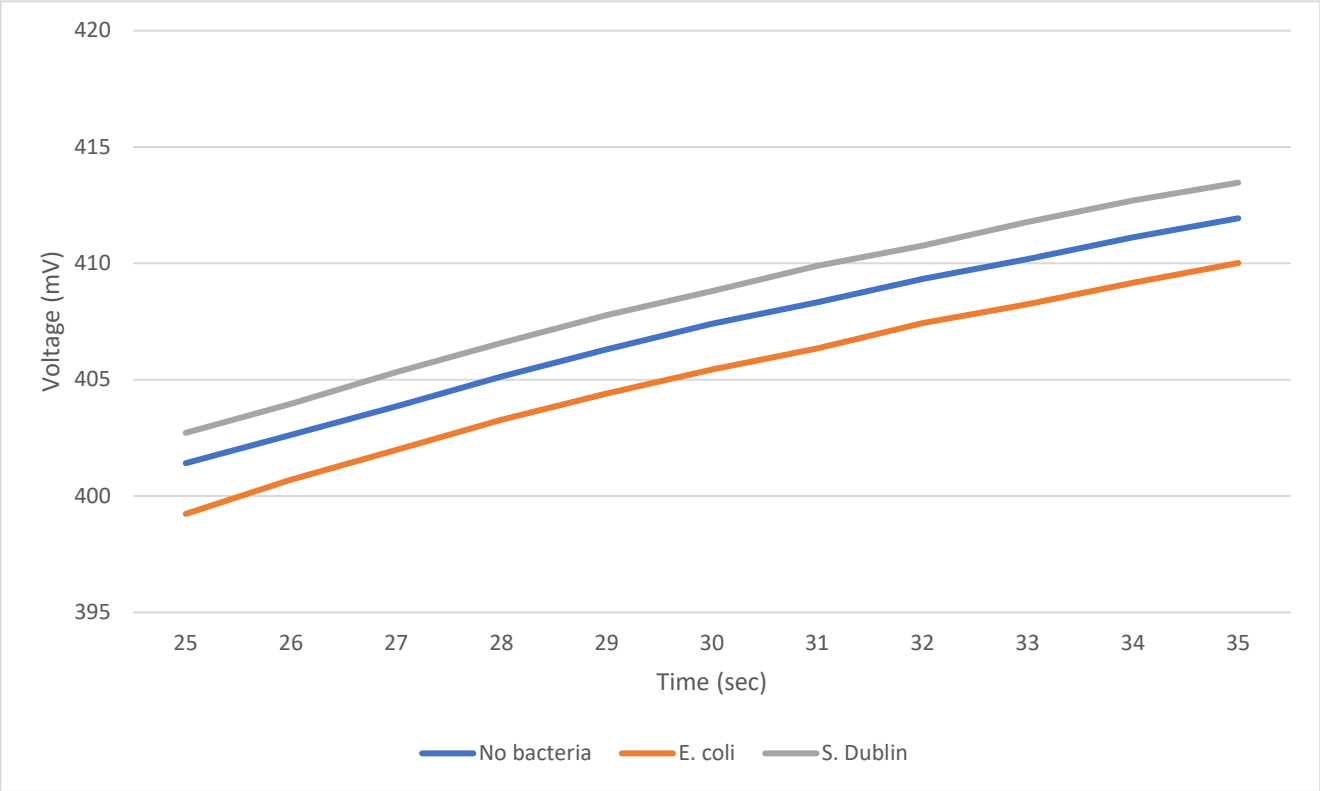
Appendix 28: An average of the optimised Vantix sandwich assay completed through calf scour, showing the 30 second timepoint on the VR2. Bacteria was diluted 1:10, from 1 OD units, in scour.



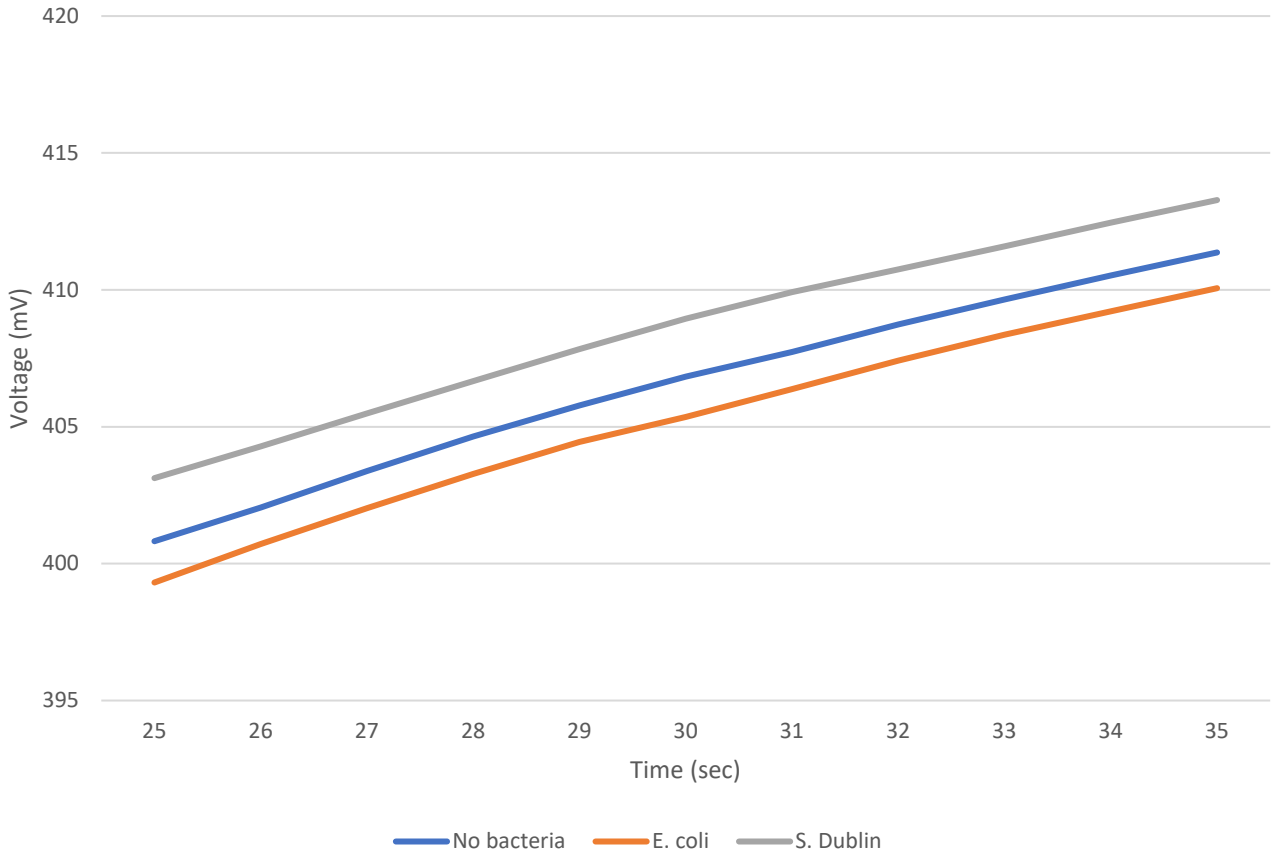
Appendix 29: An average of the optimised Vantix sandwich assay completed through calf scour, showing the 30sec timepoint on the VR2. Bacteria was diluted 1:10, from 0.5 OD units, in scour.



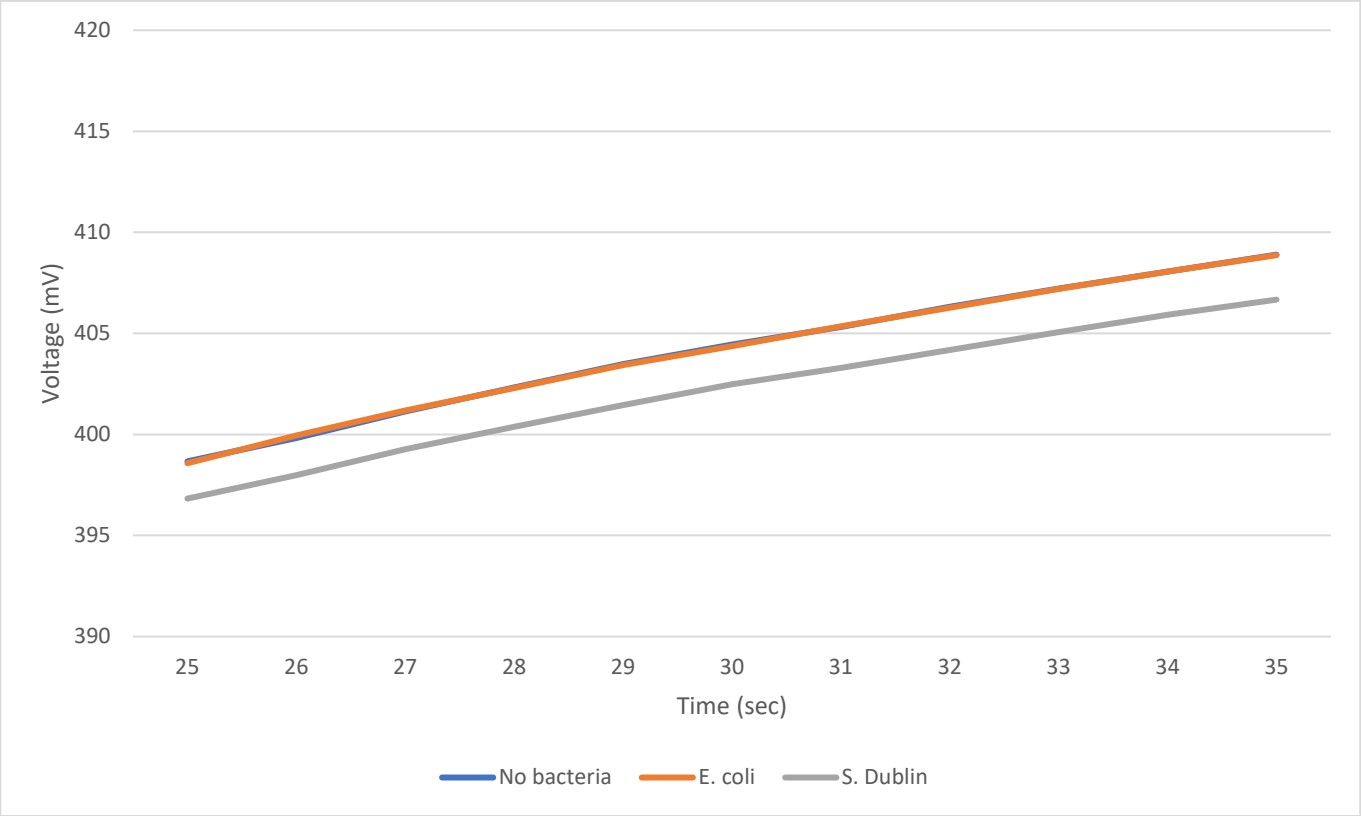
Appendix 30: An average of the optimised Vantix sandwich assay completed through calf scour, showing the 30sec timepoint on the VR2. Bacteria was diluted 1:10, from 0.25 OD units, in scour.



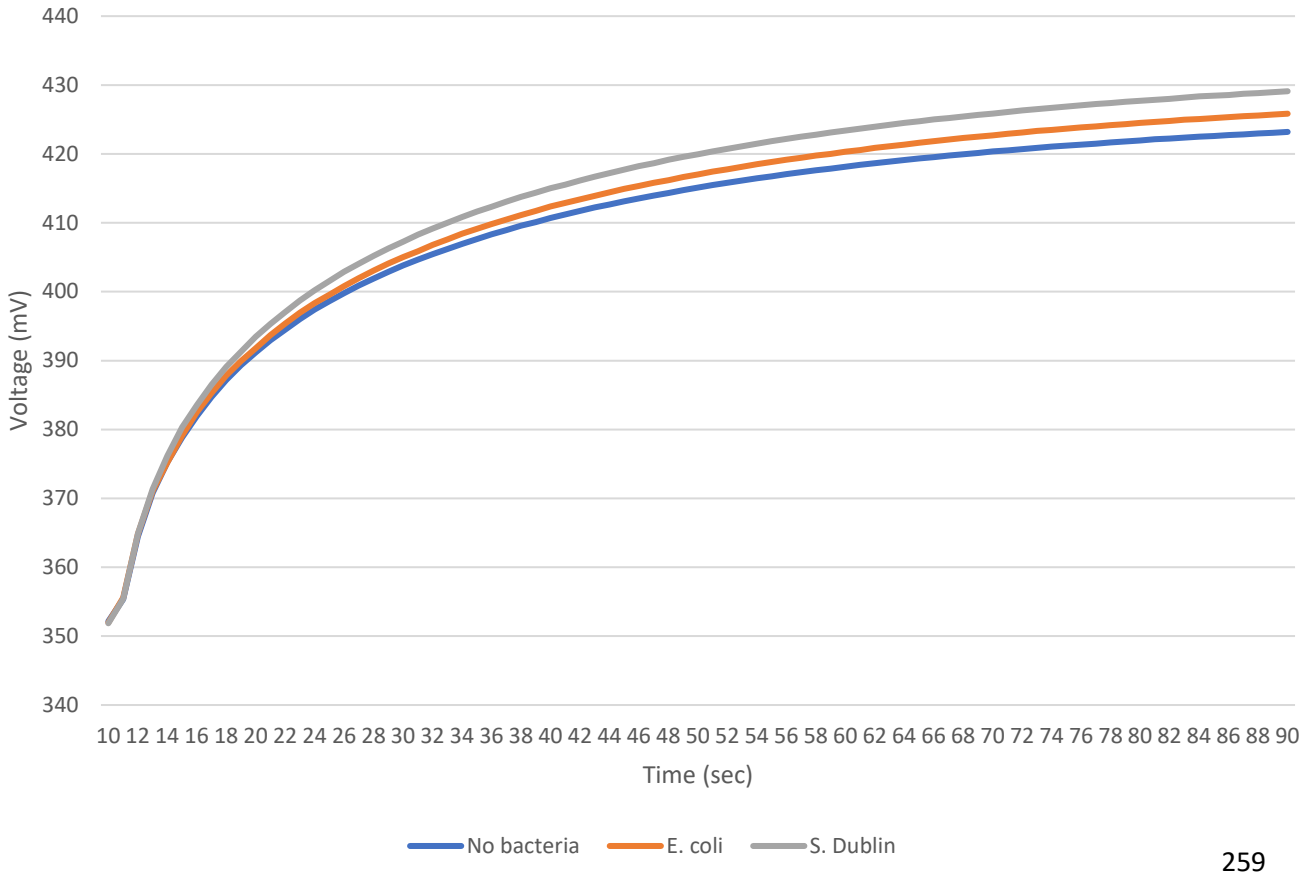
Appendix 31: An average of the optimised Vantix sandwich assay completed through calf scour, showing the 30sec timepoint on the VR2. Bacteria was diluted 1:10, from 0.125 OD units, in scour.



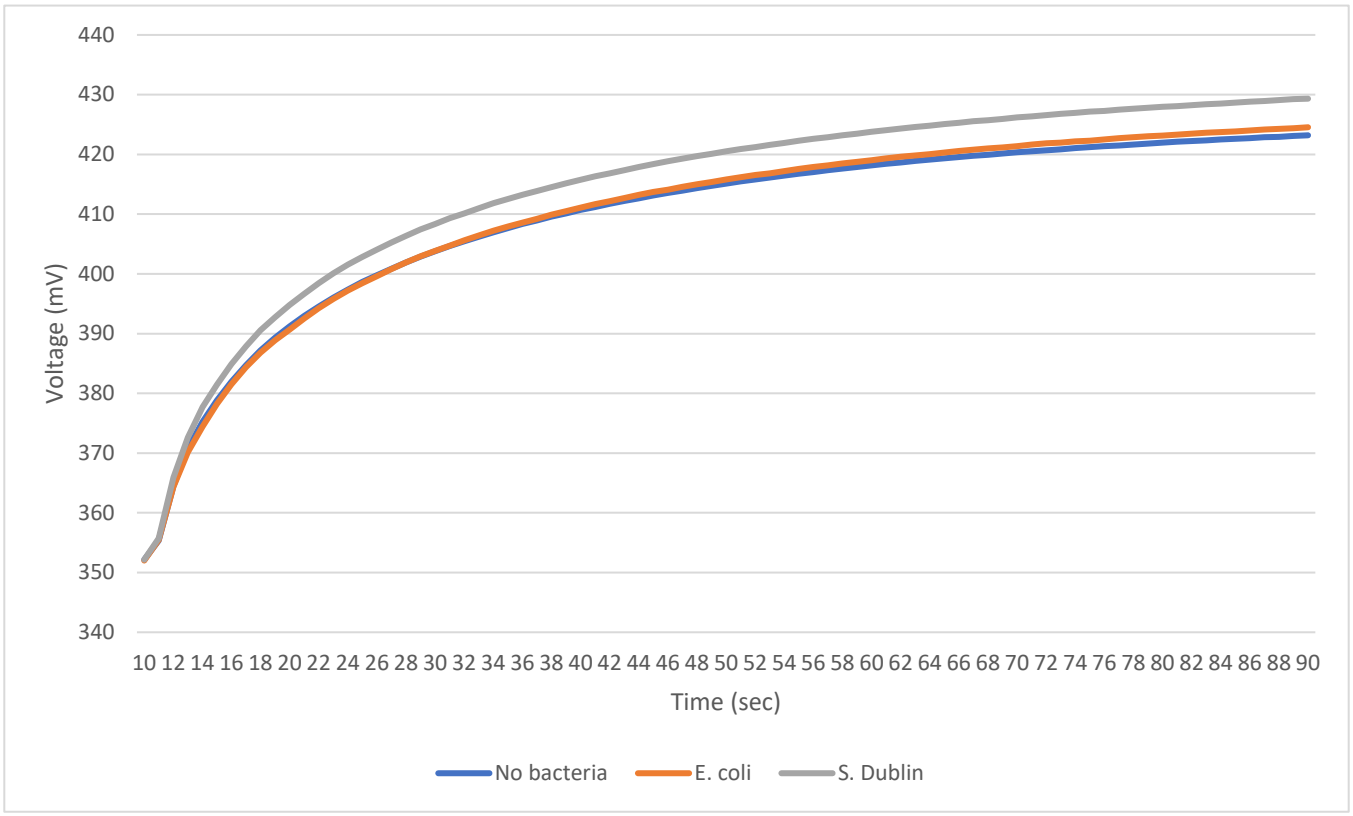
Appendix 32: An average of the optimised Vantix sandwich assay completed through calf scour, showing the 30sec timepoint on the VR2. Bacteria was diluted 1:10, from 0.06 OD units, in scour.



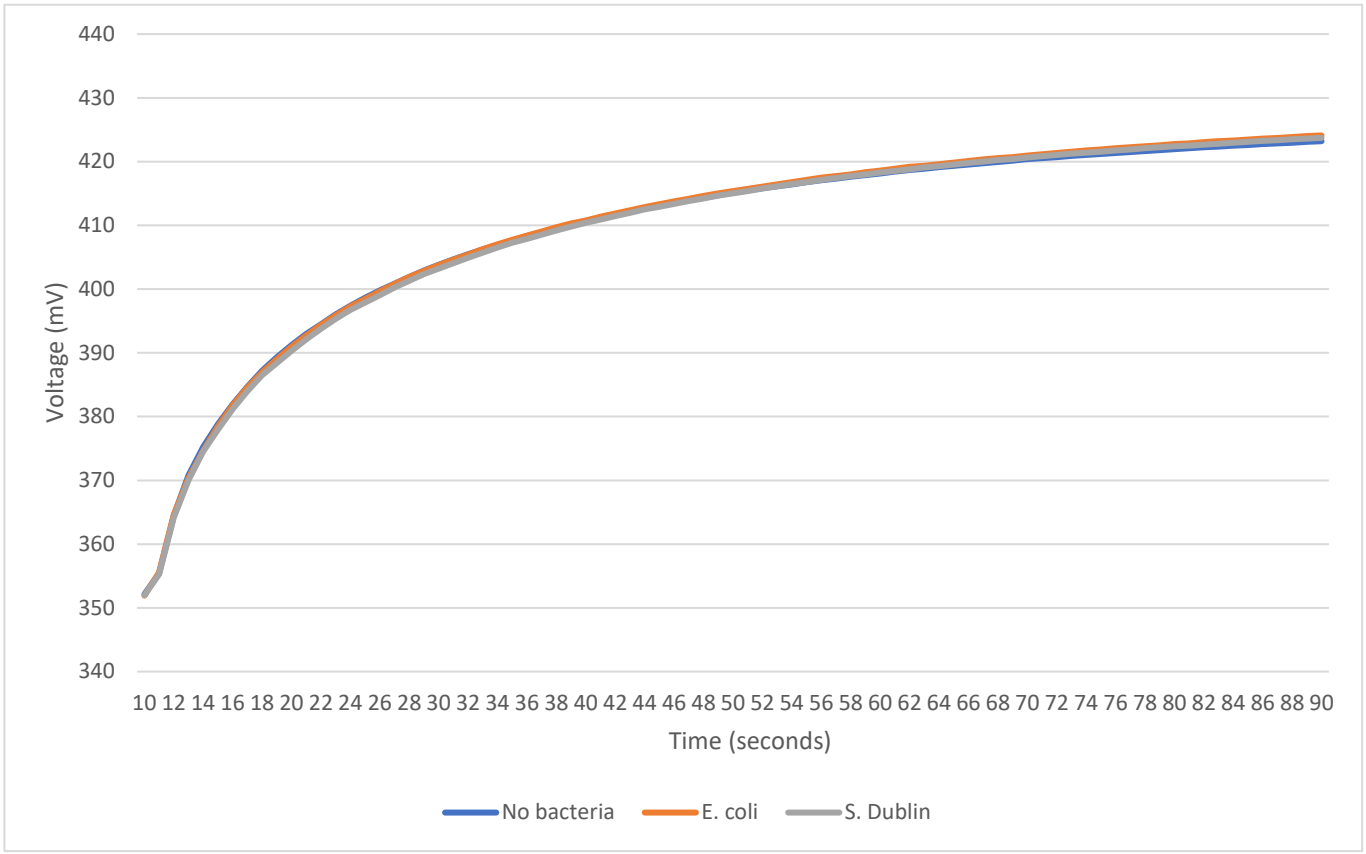
Appendix 33: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted 1:10, from 1 OD units, in scour.



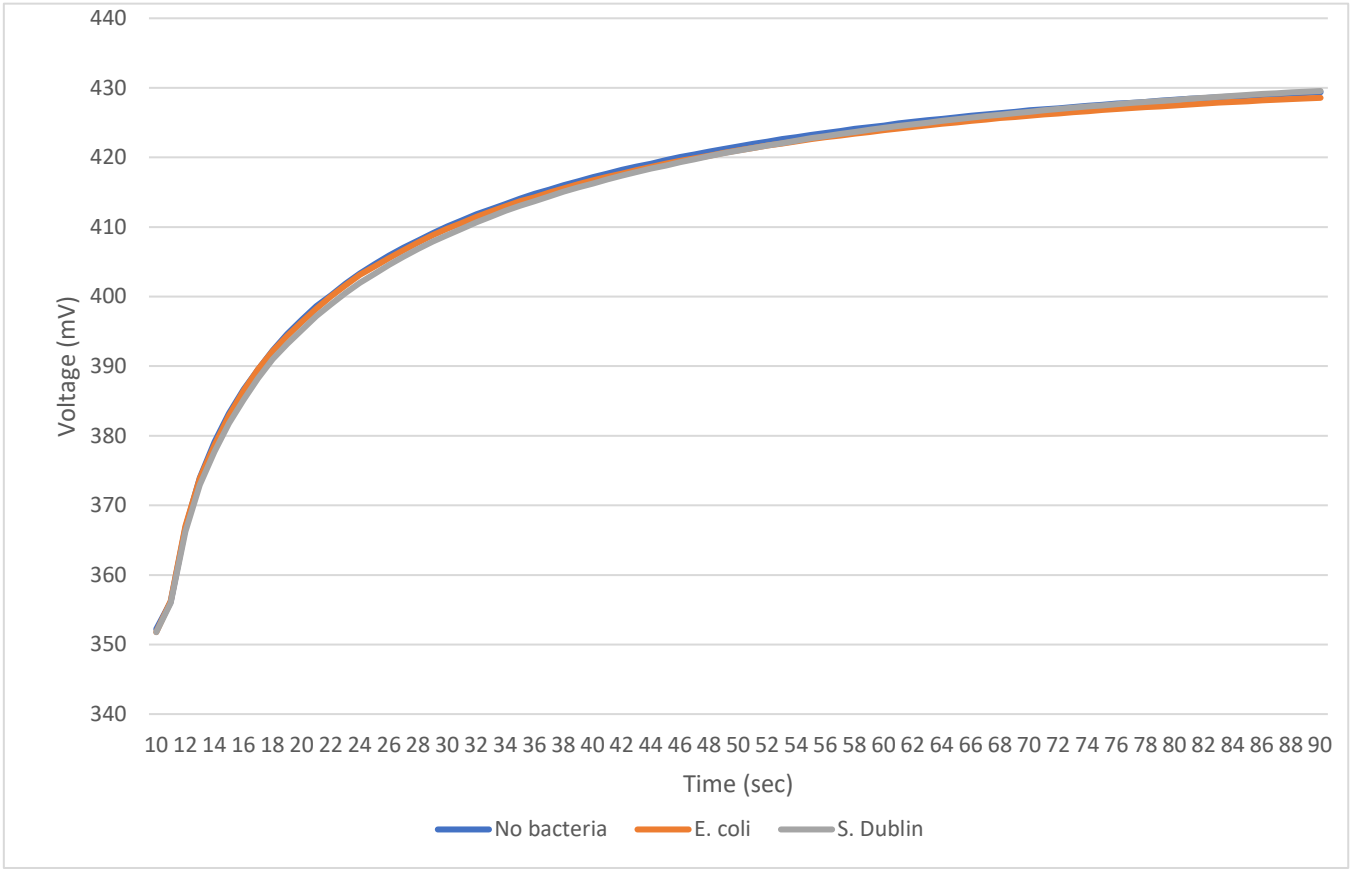
Appendix 34: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted 1:10, from 0.25 OD units, in scour.



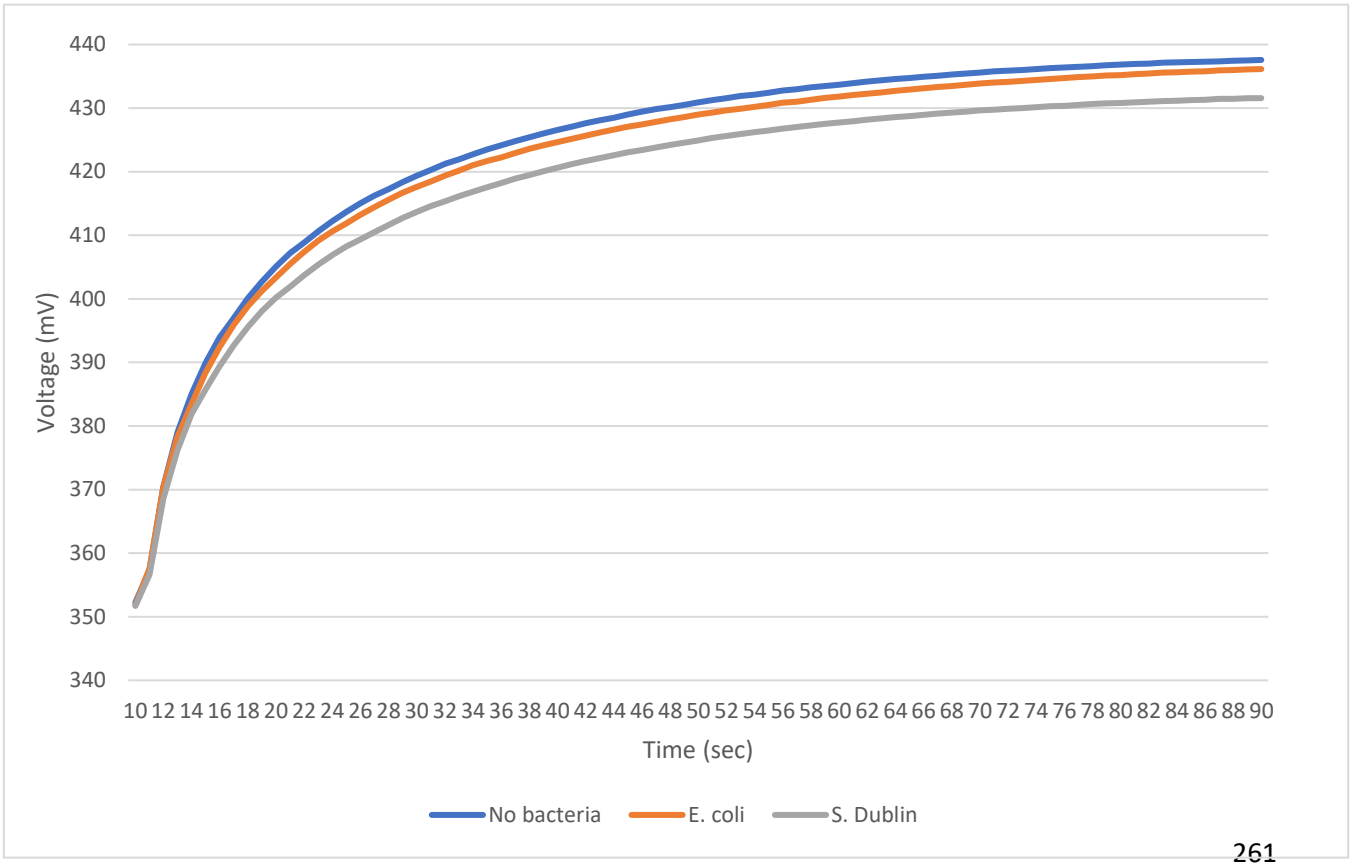
Appendix 35: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted 1:10, from 0.125 OD units, in scour.



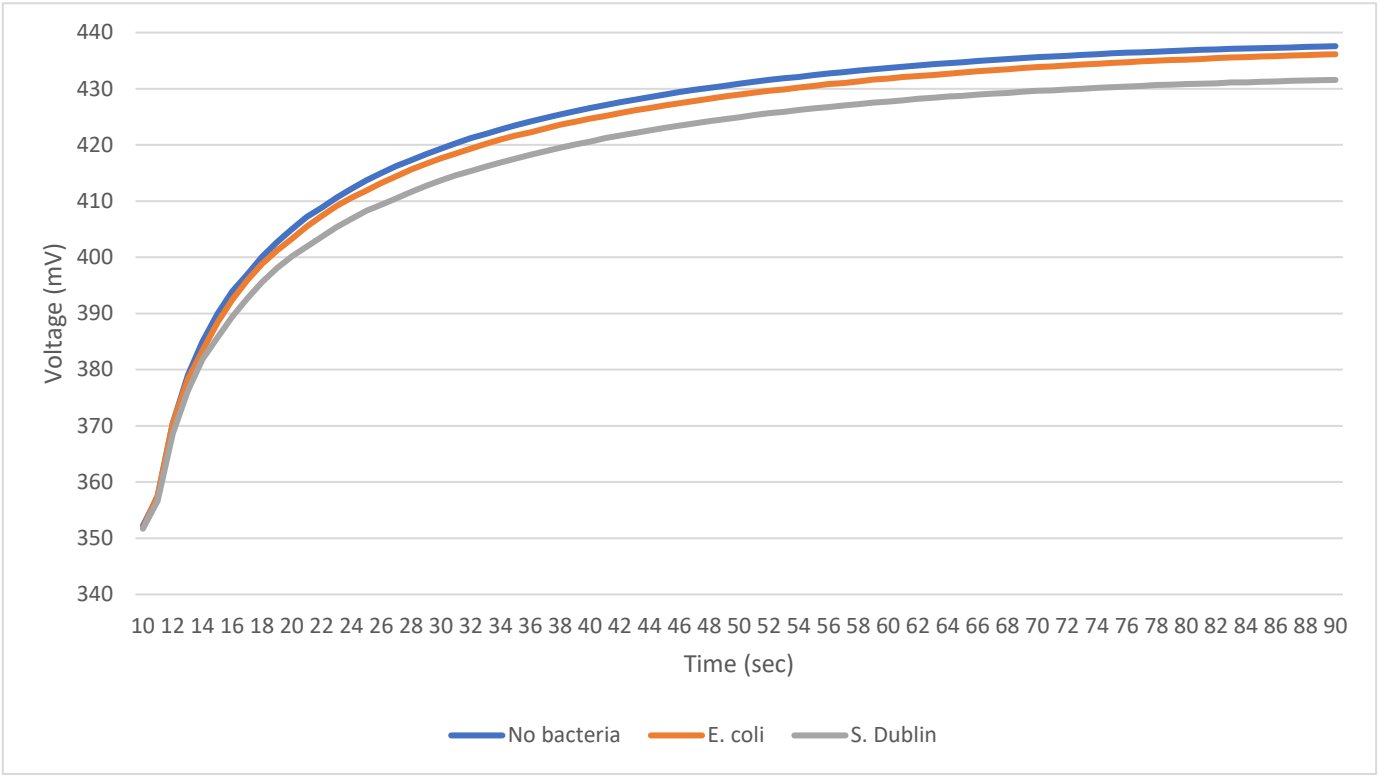
Appendix 36: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted 1:10, from 0.06 OD units, in scour.



Appendix 37: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted 1:10, from 0.03 OD units, in scour.



Appendix 38: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted 1:10, from 0.015 OD units, in scour.



Appendix 39: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted 1:10, from 0.007 OD units, in scour.