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A rapid detection method for *Salmonella* in calves with Scour

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1. 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, in order to reduce animal suffering and mortality rate. 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.

2. Introduction

Salmonellosis, 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). *Salmonella enterica* are bacteria, the causative agent of Salmonellosis and are commonly found in the environment. *Salmonella* species (sp.) can be transferred from animals to humans (zoonosis) and have a broad host range across multiple animal species (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). *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).

Humans can be quite susceptible to gastroenteritis, of which *Salmonella* sp. can be a causative agent, with an elevated incidence in many countries caused by foodborne pathogens such as *Salmonella* sp., *Campylobacter coli,* and *Escherichia coli* (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). Costa *et al.* (2012) suggested that cattle are one of the most common sources of infection for human salmonellosis and 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).

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.

2.1. 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 genetic complement and the fitness of both the host and infecting *Salmonella* serovar will determine the outcome of the infection. Dependant 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 *Salmonella enterica* serovar Dublin (*S.* Dublin) and *Salmonella enterica* serovar Typhimurium (*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 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).

There is some similarity between the two *Salmonella* serotypes, 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 gut; with symptoms which are common with an acute *S*. Dublin infection (Table 2.1; Frost *et al.,* 1997).

S. Dublin is host adapted to cattle, resulting in a variety of symptoms (Table 2.1; 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 in cattle, spreading beyond the gut (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 2.1: 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 or 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

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). 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. 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 differentials 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. 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.

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.

2.1.1. Aetiological agents of calf scour and antibiotic stewardship

Diarrhoea is the most common symptom of salmonellosis in cattle; however, it is not the only etiological 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 with focus several aspect 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* spp. 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 the efficacy of currently available *Salmonella* vaccines is not optimal, to ensure that the health and welfare impact of salmonellae is reduced quick, reliable diagnostics allowing for targeted treatment is required. *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.

2.2. 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 (Falkenhorst *et al.*, 2013; Nielsen, 2013; Vo *et al.*, 2006). However, bacterial culture is time consuming, has relatively low sensitivity and is laborious (Jadidi *et al.*, 2012; Falkenhorst *et al.*, 2013; Nyman *et al.*, 2013; Nielsen, 2012; Mateus *et al.*, 2008). Several factors, including competing bacteria, 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 when used to diagnose carrier animals with intermittent shedding (16-20%; 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).

2.2.1. Rapid diagnostics: nucleic acid amplification

Nucleic acid amplification, is a technique that detects and organism by targeting its DNA and replicating it. Nucleic acid amplification is a valuable tool in the diagnosis of infectious diseases and among various 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. Due to the widespread challenges presented by *Salmonella* infection, the need for the development of rapid and sensitive methods for diagnosis is clear. Additionally, cost effective tools would aid in the surveillance and control of *Salmonella* in cattle (Jadidi *et al.*, 2012; Nyman *et al.*, 2013; Moore and Feist, 2006; Nielsen, 2012). Nielsen (2012) suggest that a cost-effective method would enable studies to include larger samples of cattle within the research area strengthening results.

Loop mediated isothermal amplification (LAMP) works at a constant temperature using a DNA strand displacement reaction (Notomi *et al.*, 2000). Nagamine *et al.* (2002) showed that LAMP enables DNA amplification in less than 30 minutes with high 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 florescent 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)

The development of a rapid detection method for *Salmonella* would enable targeted treatment to increase animal health and welfare. Additionally, a rapid detection method would also reduce the economic and welfare costs for the farming industry, reduce the risk of infection to humans and support surveillance and control methods for *Salmonella* sp. Ideally the method would be cheap, specific, sensitive and robust. 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.

2.2.2. 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 enzyme-linked immuno-sorbent assays (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. 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 (Kokkinos *et al*., 2016; Holford *et al*., 2012; Konchi *et al*., 2007; Bahadir and Sezginturk, 2015). 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 were set;

- 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 are capable of detection multiple *Salmonella* serovars, specifically *S.* Dublin as it is host adapted to cattle, with high sensitivity and specificity
- To ensure that both diagnostic assays are robust through calf scour as a test sample
- To optimise both diagnostic methods to allow for rapid detection, without losing sensitivity

3. Materials and methods

3.1. 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.

3.1.1. 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.

3.1.2. 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.

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 section 2.1.2.1, Public Health England (PHE), 2014).

3.1.3. 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.

3.1.4. Isolates used for LAMP assays 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, *S.* Mbandaka, *S.* Montevideo, *S.* Typhimurium (strain: DT104) and *S.* Newport. These clinical strains were isolated from cattle were procured in Dec 2015 isolates. 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

3.2. Bioinformatic methods

3.2.1. Genomes used within Mauve genome alignments

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 (Table 3.1)

Table 3.1: 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	CP 007270
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

3.2.2. Genome alignment using Mauve

Genomes 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 to 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.

3.2.3. Specificity testing of genes and primers

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.

Definitions of conservation and specificity for genes

Genes 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.

3.2.4. Primer generation for nucleic amplification techniques

The genes targeted for primer generation 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.

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 3.3.3) and used downstream within PCR assays (Table 3.4).

LAMP primer generation using PrimerExplorer V. 4

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 3.2). 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 3.2).

Table 3.2. 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 3.2.4, 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 3.3.3) and used downstream within LAMP assays (Table 3.5).

Specificity testing of primers

Primer sets were assessed as per section 3.2.3, 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.

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.

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.

3.3. Nucleic amplification for detection of *Salmonella* sp.

3.3.1. 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. Optical densities (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 3.3., the purified DNA was stored at -20°C.

Table 3.3: 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	Ensures accurate ratio measurements	-
320	≤0.1	Indicates background absorbance	Turbidity, stray particulates or high absorbance buffer solutions

3.3.2. Gel electrophoresis for visualisation of nucleic amplification products

To visualise nucleic acid amplification results, 2% agarose gels were made by heating 1.5g of agarose with 75ml of 1x TBE buffer. Once cooled slightly, 9ul 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 was 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. 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.

TBE (Tris/Borate/EDTA) buffer

TBE buffer was prepared at10x concentration by adding 108g Tris Base, 55g Boric Acid and 7.5g EDTA disodium salt to 800ml of distilled water. The pH was then adjusted to 8.0 and water was added to make a final volume of 1L. A 1 in 10 dilution was then completed to get the working concentration of TBE.

3.3.3. 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 RNAase-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 3.4: Oligonucleotides used within polymerase chain reactions.

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 3.5: 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

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

Gel electrophoresis was used to visualise all PCR results, see 3.2.2. 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.

3.4.1. 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 (Table 3.4) 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 3.6, to a 0.2ml microcentrifuge tube, and added to a thermal cycler set with the cycling parameters in Table 3.7 to enable amplification.

Table 3.6: 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 3.7: 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

3.4.2. 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 completed using the PCR primers developed using Primer BLAST (Table 3.4) 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 3.8. Assay tubes were added to the thermal cycler and products were amplified as per the cycling conditions in Table 3.9.

Table 3.8: 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 3.9: 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.

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

All LAMP products were visualised using gel electrophoresis, see 3.3.2, 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 was divided by Avogados 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.

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

To determine whether the LAMP primers generated using PrimerExplorer V.4., Table 3.5, 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 3.10. 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.

Reagents were added to 0.2ml microcentrifuge tubes, as per Table 3.11, 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 3.10: 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 3.11: 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

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

Visualisation of LAMP product via turbidity

To optimise the Optigene protocol, section 3.5.1, methodology from Mori *et al.* (2001) was adapted, Table 3.12, 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 3.13. Reagents were added to 0.2ml microcentrifuge tubes, as per Table 3.12, 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 3.12: LAMP protocol optimised from table 3.10 to allow for the visual observation of turbidity

*for primer mix see table 3.13

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 3.13: The protocol used to generate the primer mix used within LAMP assays optimised for visual turbidity, see table 3.12. 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**

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 3.5.1. Reagents were added to 0.2ml microcentrifuge tubes as seen Table 3.14 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 3.14A.

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

Table 3.14: 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 3.13

Table 3.14A: The dyes used to develop a method of visualisation of the LAMP assay within this study, their mechanism 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	0.04mg/ml	Dark pink to light, bright pink	Yes
SYBR Green I	DNA intercalating	400x concentration	None	Yes
SYBR Safe	DNA intercalating	400x concentration	None	Yes

Fluorescent assays for LAMP product visualisation

To enable visualisation of the LAMP products several dyes that emit florescence when intercalated with DNA were tested. Reagents were added to 0.2ml microcentrifuge tubes as seen Table 3.14. Reaction tubes were placed in a hotplate at 65°C for an hour and checked for florescence under a UV lamp, when compared to the no template DNA control, every 5mins. The reaction was terminated at 85°C for 5mins. For dye concentrations see Table 3.14A.

Dyes tested: Propidium Iodide, SYBR Safe and SYBR green

3.6. 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 3.15). Antibodies were stored long term at -20°C, aliquots for use were stored at 4°C for 1 week.

Table 3.15: 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	Antibody is 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)	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.*

3.6.1. 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 the 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.

3.6.2. Antibody dilution for use within immunoassays

Antibodies were diluted with carbonate bicarbonate buffer (section 3.7.1) as needed for use within immunoassays; see sections 3.8 - 3.9.

3.7. Buffers and substrates for use with immunoassays

3.7.1. 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.

3.7.2. 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).

3.7.3. 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 sulfuric 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.

3.8. 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*

3.8.1. 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.

3.8.2. 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 100ul 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 3.7.3) was added to each well. Plates were developed at 10 minutes and the reaction was stopped by adding 2M sulphuric acid. Optical density was measured using a 96well plate reader at 450nm.

3.8.3. Optimisation of Direct ELISA protocol

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).

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 (Figure 3.1). Using a multichannel pipette, 50µl from column 1 was removed and mixed via pipetting in column 2. Tips were changed, and 50µl from column 2 was removed and mixed by pipetting within column 3. This process was repeated across the plate. From column 12, 50µul of solution was removed and discarded (Figure 3.1). The plate was incubated for 16-18hr at 37°C. Plates were then processed as described in section 3.8.2, 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

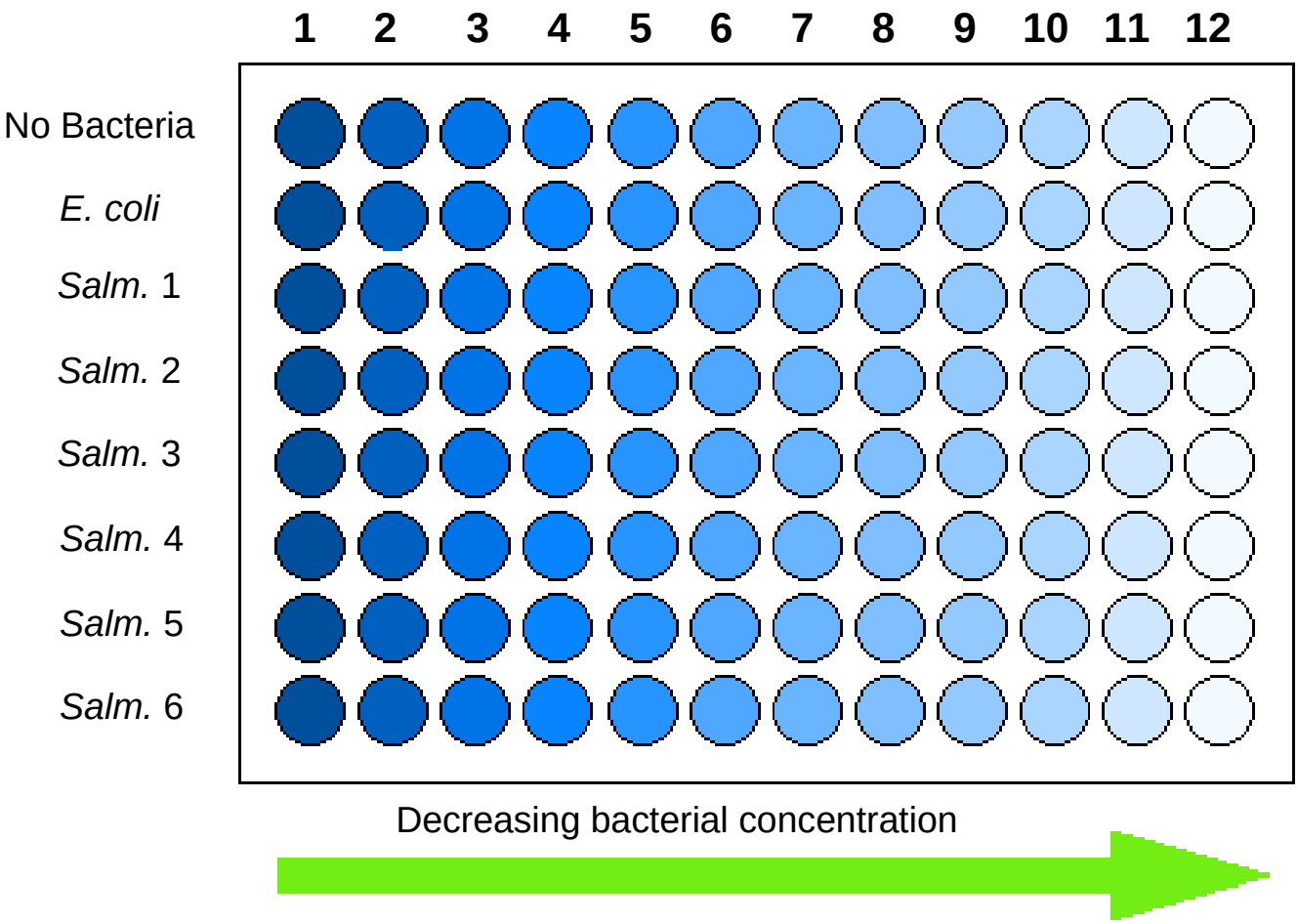

Figure 3.1: How a 96-well plate would be inoculated by serial dilution to allow for optimisation of multiple ELISA steps.

Optimisation of wash steps

To determine the effect of the wash step on the efficacy of Direct-ELISA, the protocol in section 3.2.8.2 was completed with the following changes to the wash steps;

- 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.
- 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

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

- Incubation at 37°C

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);

- Dilution at 1:100
- Dilution at 1:500

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

All immunoassays were completed in triplicate unless otherwise stated.

3.9.1. 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 optical density 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

3.9.2. Assays using Vantix Diagnostic Research Tool 1 (VR1)

For initial assay development, the original Vantix Diagnostic Research Tool (VR1) was used (Figure 3.2).

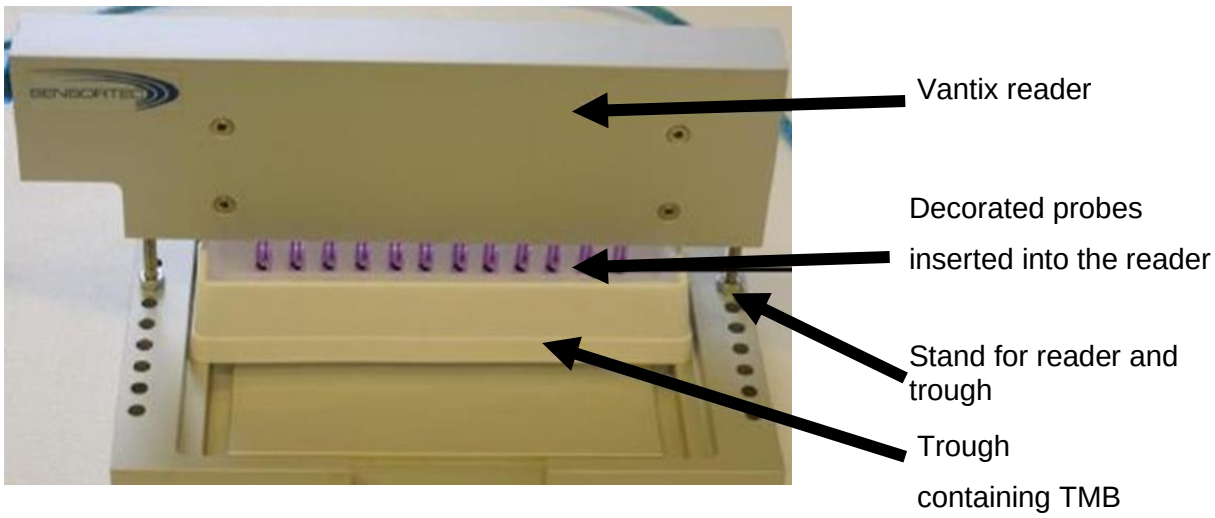

Figure 3.2: The Vantix Diagnostic Research Tool (VR1), which detects the potentiometric response of the assay on the decorated probes.

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 3.3). 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.

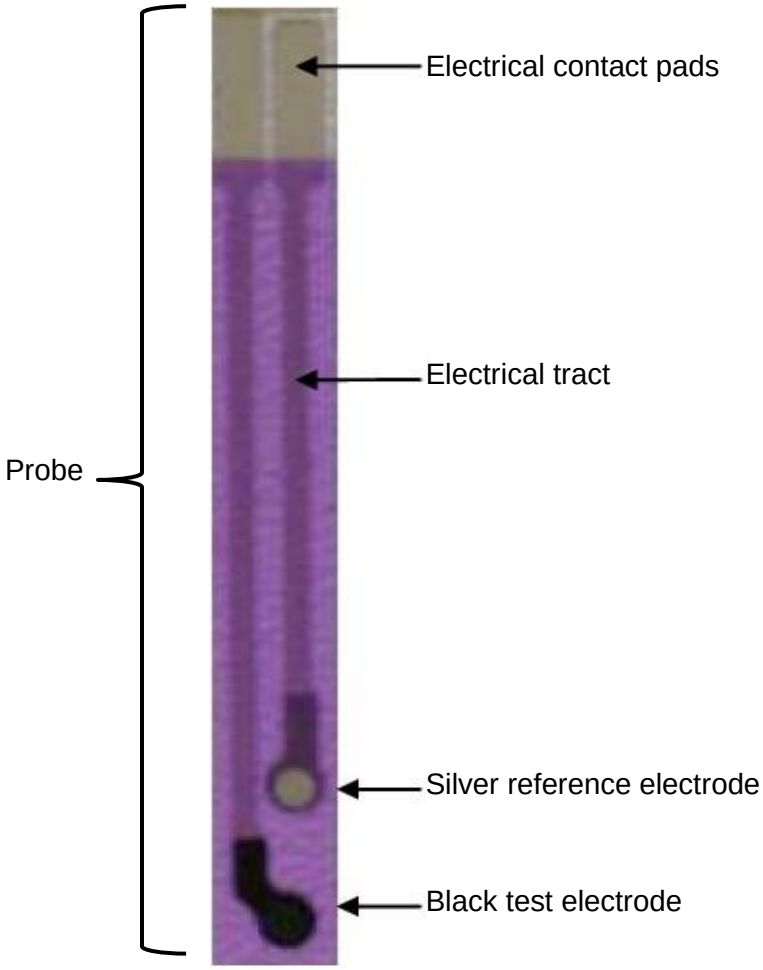

Figure 3.3: An example of a VR1 probe. The potentiometric signal generated by the assays is detected by the difference in voltage between the two electrodes (Image adapted from Cork *et al*., 2013).

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 3.15.

Cultures were prepared as stated in 3.9. On black test electrode, 3ul 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 3ul were added and probes were incubated at 37°C for 2hrs. Washing was repeated, and black electrodes were blocked with 3ul of PBS containing 0.1% skimmed milk powder (w/v) for 1 hour at 37°C. Probes were washed and then aliquots of 3ul 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.

3.9.3. 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 3.15. The Vantix Diagnostic Research Tool (VR2) was used in the following assays (Figure 3.4).

Figure 3.4: 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).

Probe preparation for assay

Probes came in pre-designed combs to fit the VR2 (Figure 3.5). 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.

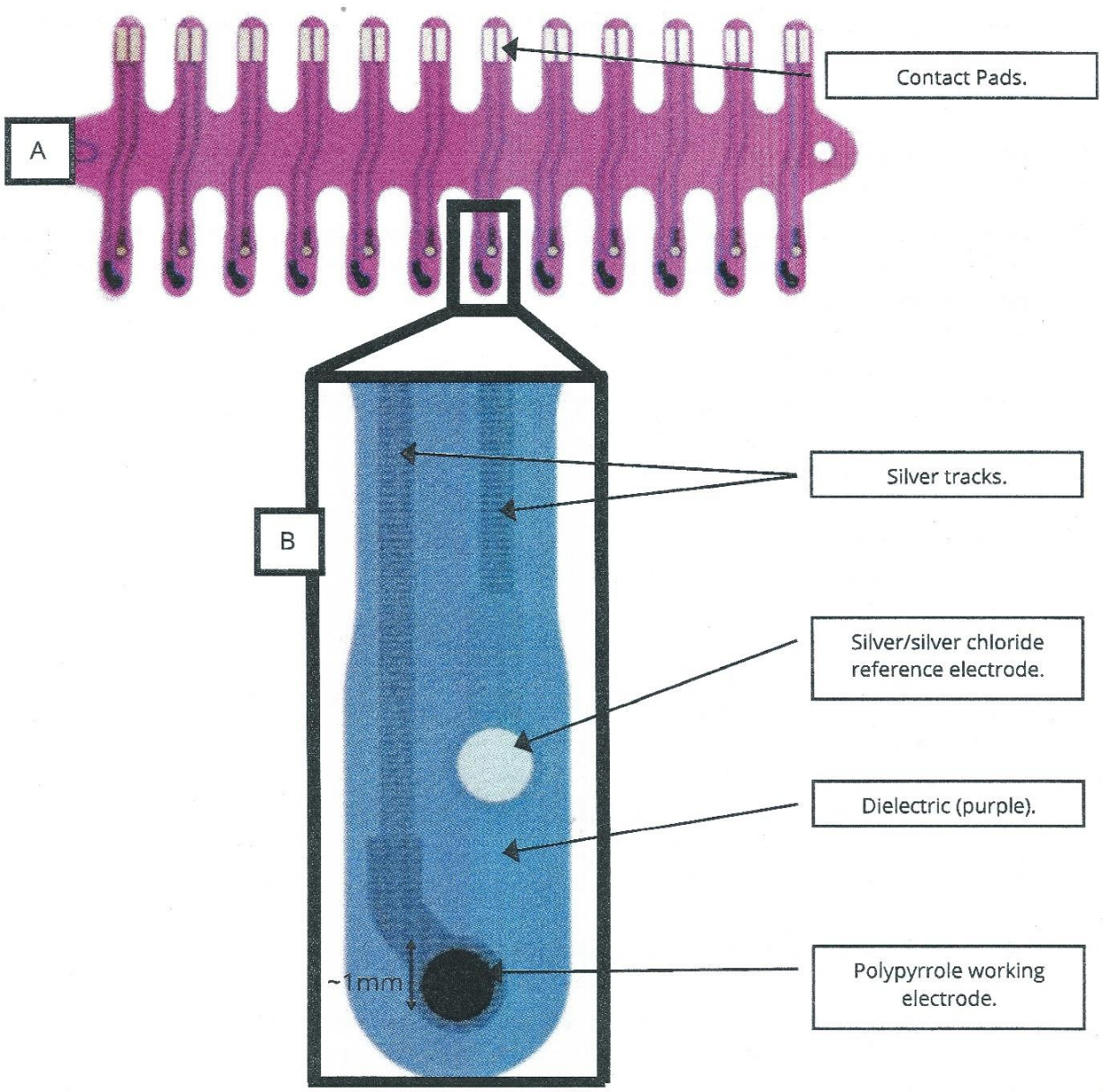

Figure 3.5: An example of a VR2 comb of probes. A = sensor comb consisting of 12 probes, B = An individual probe showing a close view of electrodes (Image taken from Vantix.com).

Sandwich Vantix assay

Cultures were prepared as stated in 3.9. On black test electrodes, 2µl of unconjugated polyclonal antibody (TRP, 1:500) was aliquoted then incubated at 37°C for 30 mins, see Figure 3.6. 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, 1:100) 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 3.7) 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.

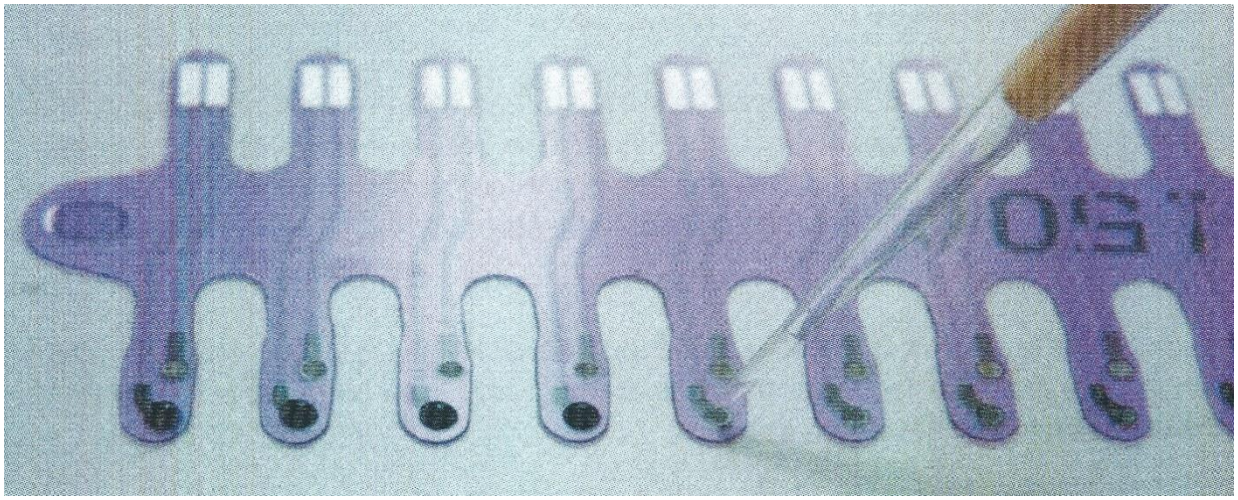

Figure 3.6: A comb of VR2 probes being decorated with an assay substrate (Image taken from Vantix.com).

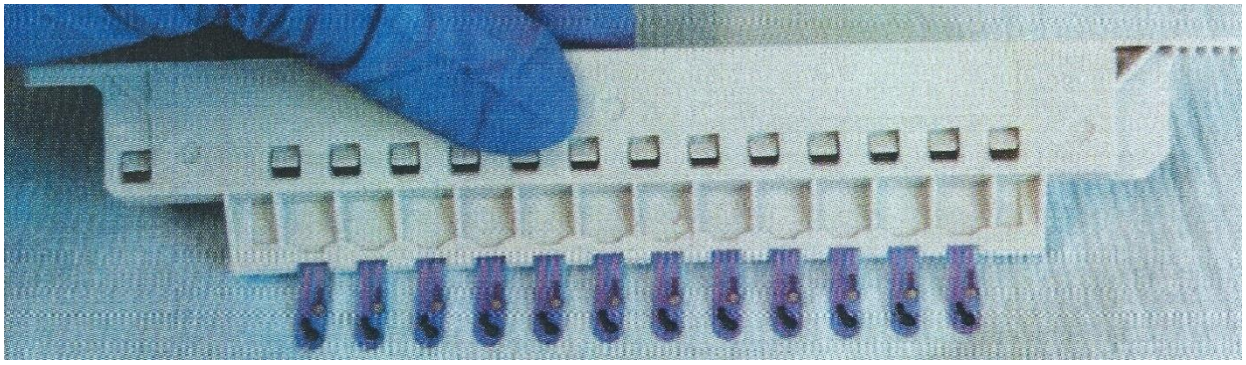

Figure 3.7: A clip containing decorated probes to be read within the VR2 reader (Image taken from Vantix.com).

Reduction in step incubation time

Sandwich assays was completed as per section ‘Sandwich Vantix Assay’, however differing incubation times were tested as per Table 3.16 to determine is sandwich assay sensitivity could be maintained through shortened incubation periods

Table 3.16: 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

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 3.9.1. 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.

Vantix sandwich assay through calf scour

Sandwich assay was completed as described in section 2.9.2.3 with the following optimisation;

- Faecal samples, containing known bacterial numbers, used in place of antigen.

Faecal samples were treated as per section 3.1.2 before use in the Vantix sandwich assay. Faecal samples were vortexed to ensure a uniform suspension of matter. Bacteria were prepared to the appropriate optical density as per the method stated in 3.8.1. 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.

4. Results

4.1. Generating a loop-mediated isothermal amplification assay to target pan-*Salmonella* genomic DNA results

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 to identify conserved gene targets across the genomes with methodology detailed in section 3.2.1. During a partial screening, 32 conserved genes were identified, 11 of which were considered highly specific to multiple *Salmonella* strains and therefore potential primer targets. 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. Using these genes as targets for primer development, 6 primer sets for polymerase chain reaction (PCR) were developed using bioinformatics, see Table 4.1.

Table 4.1: PCR primer sets produced for the detection of *Salmonella* sp. Key: bp = base pair

Gene Target	Primer set lab reference	Product Length (bp)	Primer type	Sequence (5'-3')
hilA	HilA_1	660	Forward	CGACAGAGCTGGACCACAAT
			Backward	TCAAGCGGGGATCCTGTTTC
	HilA_2	344	Forward	ACCAACCCGCTTCTCTCTTG
			Backward	ATTGTGGTCCAGCTCTGTCG
orgA	OrgA_1	384	Forward	GCGGCGGCAAATGAGTTAAT
			Backward	AGCATCCTGCTTCAATGCCT
	OrgA_2	437	Forward	TATCCATCCTCAGCGGTTGC
			Backward	CCTGCTTCAATGCCTCCTCA
bapA	BapA_1	425	Forward	CGGTGAATTCGTCGTTACGC
			Backward	GATCGACAGTGATCCCGACC
	BapA_2	591	Forward	ATCGGCAATAATGGCGCAAC
			Backward	GATTTCATTGACGACGGGCG

To determine whether the target genes could be used to detect multiple *Salmonella* serovars, a PCR protocol was generated and optimised for the developed primer sets. 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., with *E. coli* as a negative control. All primer sets detected *S.* Dublin, with orgA_1 detecting all *Salmonella* serovars tested (Table 4.2, Figure X). *S.* Bovismorbificans was the least detected serovar, only detected by hilA_2 and orgA_1 (Table 4.2).

Most primer sets detected 4 out of 6 *Salmonella* serovars tested, accept bapA_1, which only detected *S.* Dublin and *S.* Typhimurium (Table 4.2).

Table 4.2: The specificity of the PCR primer sets, using the optimal primer volume and annealing temperature for each set when targeting *S.* Dublin, using HotStarTaq PCR protocol 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

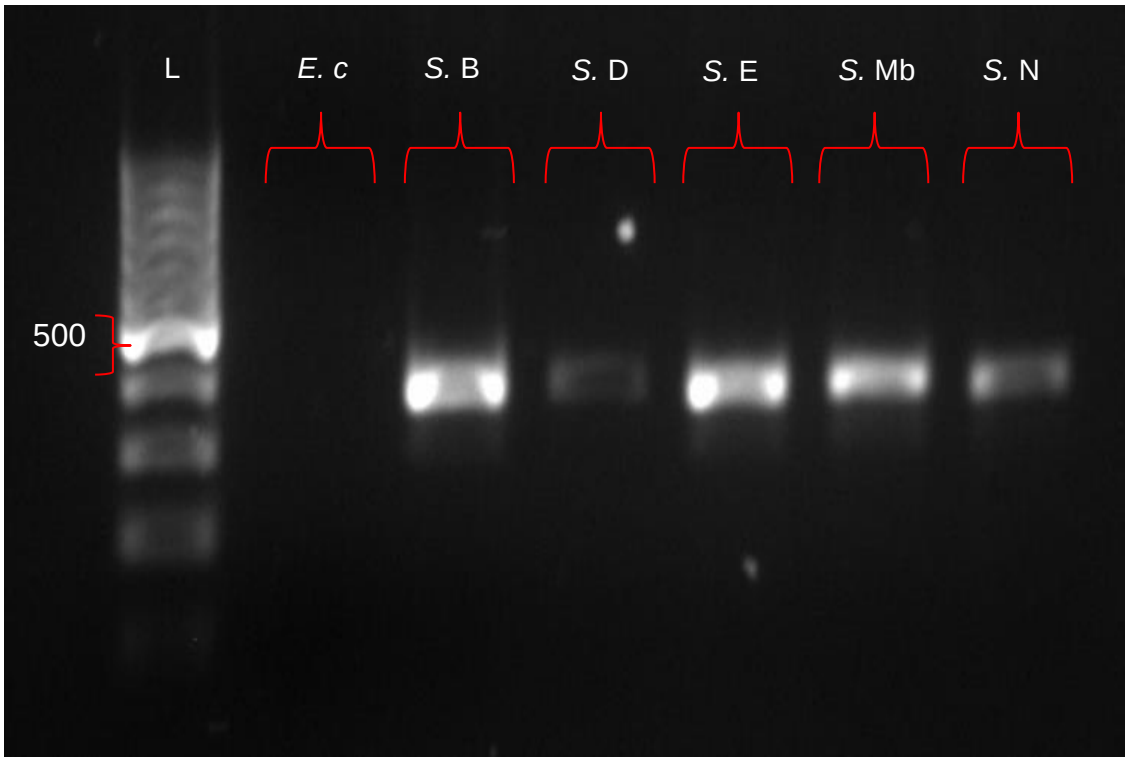

Figure 4.1: The amplification results of optimised HotStarTaq PCR method for orgA1 primer set against a selection of *Salmonella* serovars.

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

As the PCR primer sets targeting *hilA*, *orgA*, and *bapA* all detected *S.* Dublin, all 3 genes were used to generate loop-mediated isothermal amplification (LAMP) primers. Using Primer Explorer V.4, the sequences targeted by the PCR primers were also targeted for the LAMP primers where applicable:

to accommodate the size of the gene sequence, *bapA* was spilt into 2,000bp sequences in the vicinity of the PCR primer sets targeting this gene. In total, 8 LAMP primer sets were generated, 2 sets targeting *hilA,* and *orgA*, and 4 sets targeting *bapA*, see Table 4.3.

Table 4.3: 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

To establish whether the LAMP primer sets generated using bioinformatic techniques (Table 4.3) could detect *Salmonella enterica* serovar Dublin (*S*. Dublin) genomic DNA, the Optigene protocol was used, as described in method section 3.5.1. All primer sets detected *S*. Dublin DNA, except bapA1.2 (Figure 4.2).

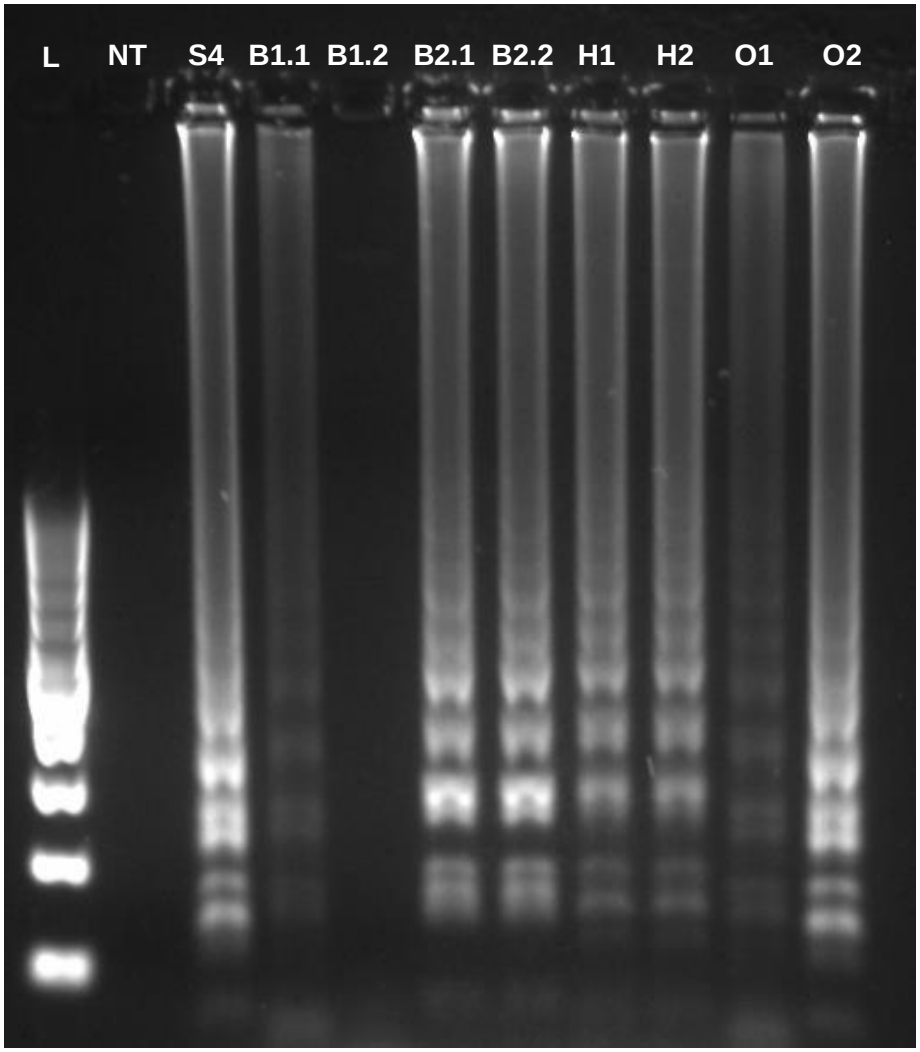

Figure 4.2: Agarose gel after electrophoresis showing LAMP assays completed using the Optigene method, against each LAMP primer set generated by bioinformatic methods, with *S*. Dublin as the target DNA.

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

To allow for visualisation of the LAMP assay results without the need to use gel electrophoresis, several protocols were developed. Visualisation of the Optigene LAMP assays was initially based upon visual inspection for turbidity, however whilst gel electrophoresis showed LAMP amplicon, no turbidity was observed. Thus, the turbidity LAMP assays were optimised. Increased concentrations of template DNA were used (1ng/µL *versus* 0.1ng/µL), producing observable amounts of product when observed via gel electrophoresis (Figure 4.3A), however little to no visual turbidity was observed within reaction tubes after centrifugation (Figure 4.3B).

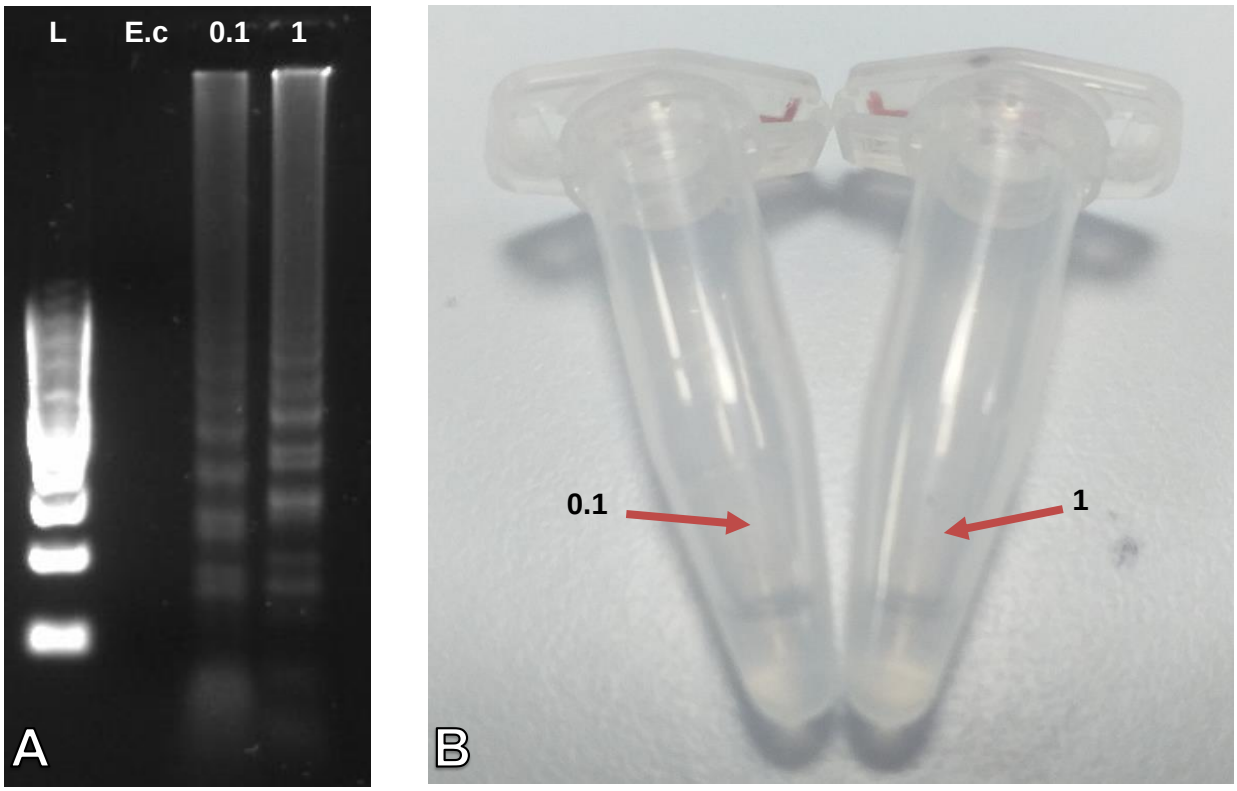

Figure 4.3: (A) Agarose gel showing turbidity LAMP assays using the orgA1 primer set with an increase in *S*. Dublin template DNA (A) with the turbidity LAMP assay reaction tubes after burst centrifuging targeting increased concentrations of *S.* Dublin DNA (B).

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)

Work then looked at adding a colorimetric dye to the LAMP assays. Several dyes were tested including; methylene blue, hydroxy naphthol blue, Nile Blue A, propidium iodide, and SYBR safe. No colour change was observed after amplification with methylene blue or hydroxy naphthol blue as a colorimetric dye, thus they were no longer tested. After optimisation, dyes were added after DNA amplification and reaction termination. With SYBR safe, no visual colour change was seen at any time point, despite amplification being apparent on the agarose gel after electrophoresis.

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 4.4), no change was seen with the LAMP primer sets generated within this study, however amplification of LAMP products can be seen via gel electrophoresis for assays tested with Nile Blue.

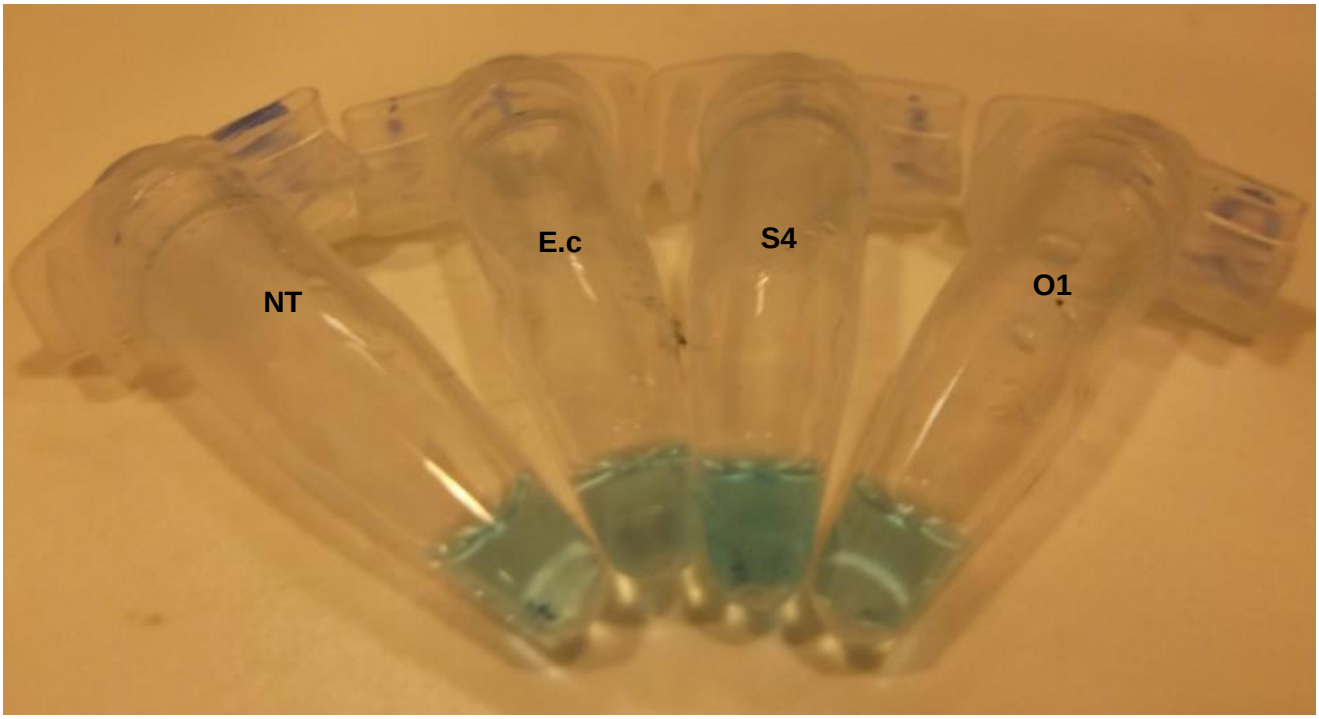

Figure 4.4: Colorimetric LAMP assay reaction tubes after 45mins amplification and Nile blue added after termination. Obvious visual colour change was only observed at 45mins within the Sal4 positive control primer set, despite product being seen from the positive control and test sample on the subsequent gel.

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

With propidium iodide, a visual change, pink to a brighter pink, could be seen between negative and positive controls at 45mins (Figure 4.5). No colour change was seen at less than 45mins despite visible amplification being apparent on the agarose gel.

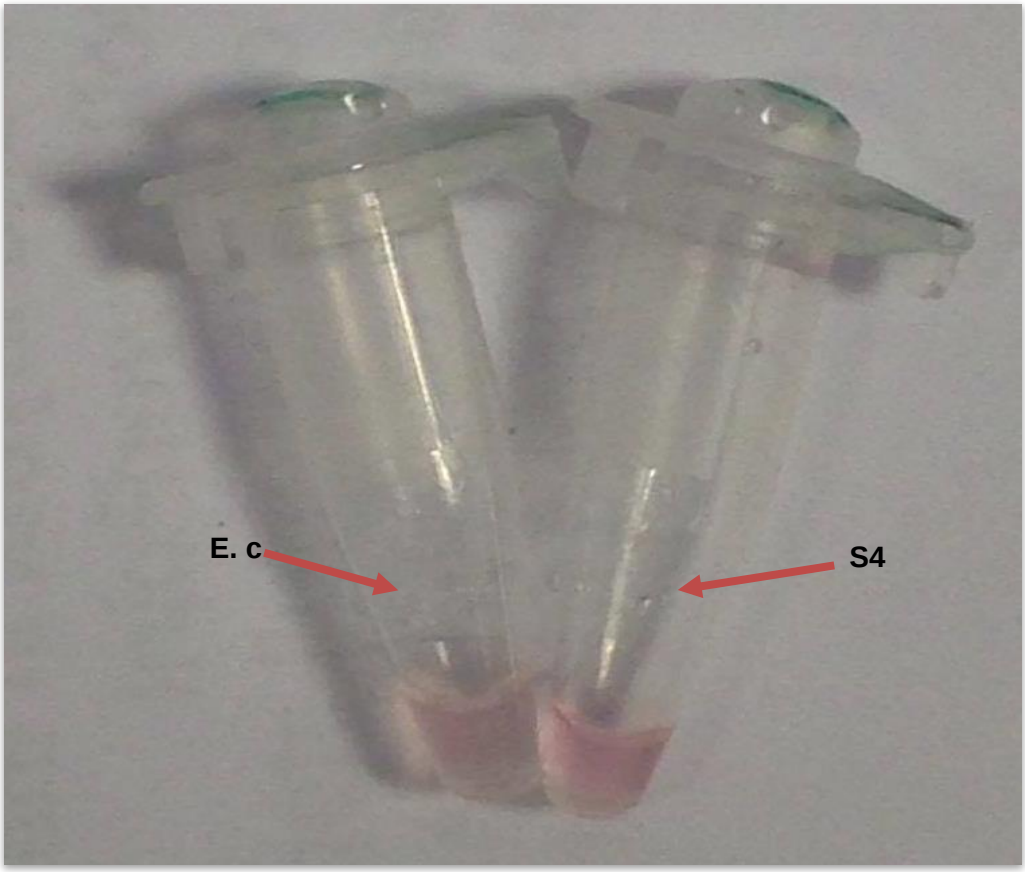

Figure 4.5: Colorimetric LAMP assay reaction tubes 45mins amplification, with propidium iodide added after termination.

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

Thus, the fluorometric LAMP assays were optimised with dyes being added after termination of the reaction. Using this protocol, amplification was observed in gels and fluorescence was seen under UV light, 3 fluorometric dyes were tested, propidium iodide, SYBR safe, and SYBR green. For each assay run, a positive fluorescence response was considered a visible change in light/colour emission under UV light. A negative fluorescene response was considered that equal to the negative controls, 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 4.6), SYBR safe produced a yellow/light orange fluorescence (Figure 4.7), and SYBR Green I showed bright green fluorescence.

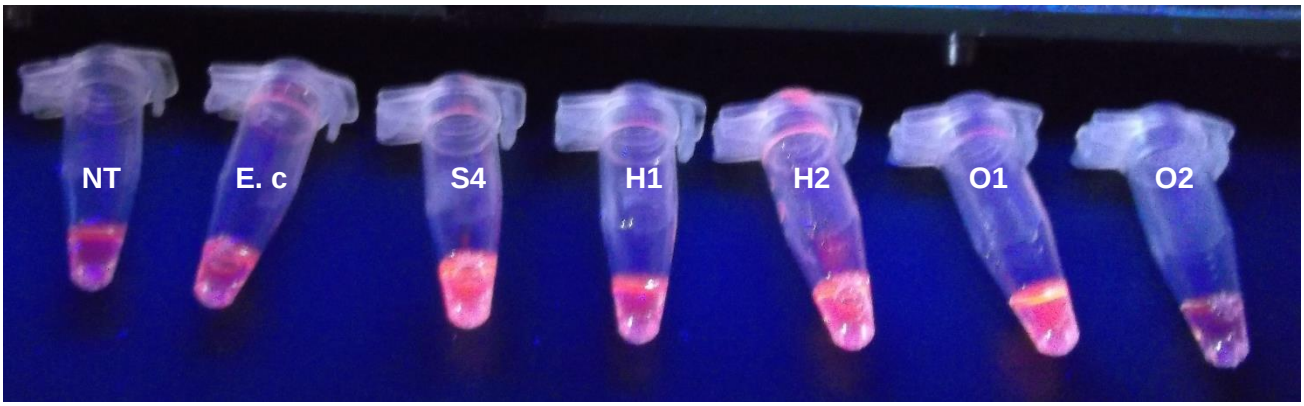

Figure 4.6: Fluorometric LAMP assay reaction tubes under UV light, after 25 minutes amplification with propidium iodide added after termination, showing clear positive signals for S4, H1, H2, and O1.

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

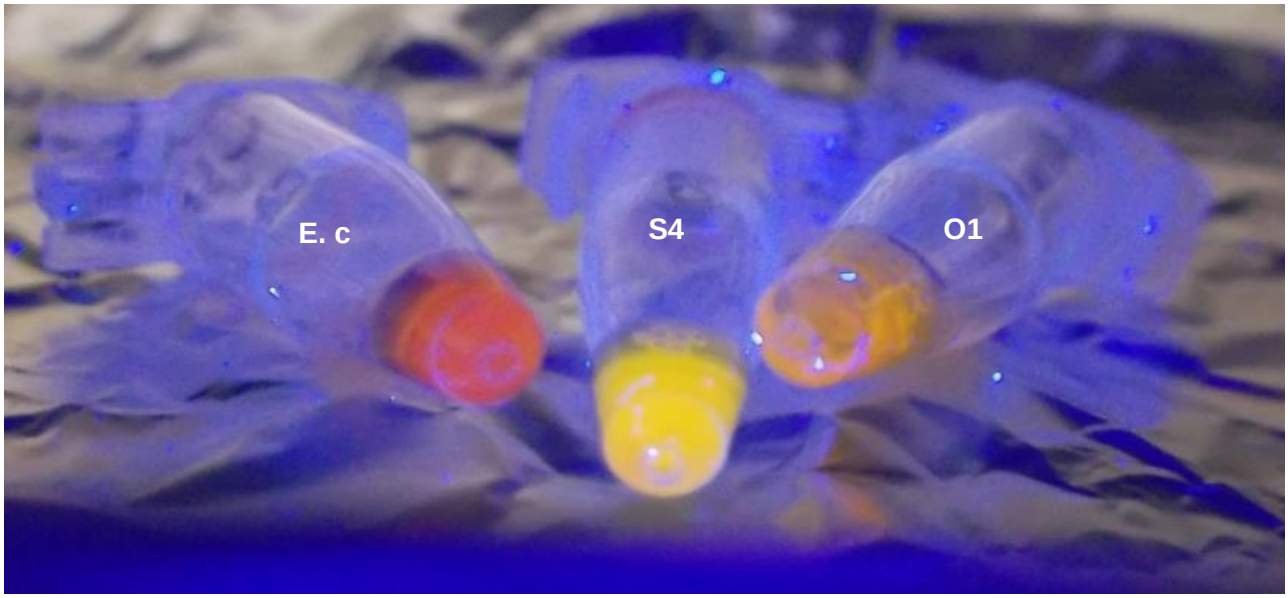

Figure 4.7: Fluorometric LAMP assay reaction tubes under UV light, after 45 minutes amplification with SYBR safe added after termination. Compared to the negative *E. coli* control, lightening of colour due to fluorescence seen for S4 and O1.

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

Agarose gels showed product that corresponded with the fluorescence observed, addition of dyes did not interfere with gel electrophoresis (Figure 4.8). In general, it was found that propidium iodide was more sensitive to low levels of amplification than SYBR safe.

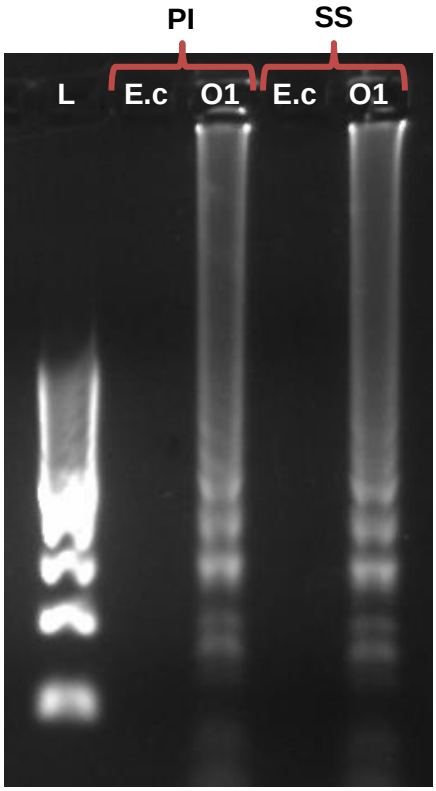

Figure 4.8: orgA1 LAMP assay reaction products on an agarose gel after 45 mins amplification and fluorometric dyes added after termination

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

Once visualisation had been achieved, the LAMP assays were optimised by reducing amplification time. Positive control primer set, Sal4, and the bapA2.1 (B2.1) primer set consistently showed strong amplification from as early as 15mins. Faint amplicon products could be seen for hilA2 (H2), orgA1 (O1) and orgA2 (O2) at 15 mins but was not consistent. At 30 minutes amplification was seen for all primer sets, with B2.1, *hilA* and *orgA* primer sets having strong amplicon products. BapA1.1 (B1.1), bapA1.2 (B1.2) and bapA2.2 (B2.2) had weak amplicon bands at 30mins.

When visualising 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 4.4 & 4.5). B1.1, B1.2, and B2.2 produced a weak response with PI at 25mins, however showed no response at 30mins (Table 4.4). With SS, B1.1, B1.2, and B2.2 produced a weak response at 30mins, with no response at time points below (Table 4.5). In general, longer amplification times generated a greater fluorescent response.

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Table 4.4: 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).

Time (mins)	Primer set										
	NT	Negative	Positive	B1.1	B1.2	B2.1	B2.2	H1	H2	O1	O2
15	0	0	0.75	0	0	0.83	0	0	0.5	0.38	0.63
20	0	0	0.5	0	0	1	0	0	0.67	0.67	0.67
25	0	0	0.75	0.17	0.17	1	0.33	0.38	0.88	0.88	0.38
30	0	0	1	0	0	1	0	0.33	0.83	0.83	0.83

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

Table 4.5: 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).

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	0.33	0	0	0	0	0
20	0	0	1	0	0	1	0	0	0.25	0.63	0.13
25	0	0	1	0	0	1	0	0.13	1	1	0.5
30	0	0	1	0.67	0.17	1	0.5	0	0.83	0.83	0.5

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

To determine the effect temperature changes would have on the efficacy of DNA amplification, amplification temperature was changed. 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. On agarose gels, after amplification at 55°C and 65°C, ladder bands were seen for B2.1, H2 and *orgA* primer sets with 65°C being optimal. At 75°C LAMP ladder patterns were weak and at 85°C no amplification was observed after electrophoresis.

When visualised with propidium iodide, strongest fluoroesence 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 and 75°C (Table 4.6). O2 showed a weak fluorescence response from 45-75°C, with no clear optimum temperature (Table 4.6). A fluorescence response was seen at only 55-65°C

for H2 (Table 4.6). O1 produced a weak fluorescence response at 45°C, and a medium response at 55-65°C.

Table 4.6: The averaged results of visible fluorescence from optimised LAMP assays performed at different temperatures.

Temperature (°C)	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	0.125	0	0.1	0.1
55	0	0	0.6	0.625	0.7	0.5	0.2
65	0	0	0.6	1	0.5	0.5	0.2
75	0	0	0.2	0.125	0	0	0.2
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)

Once optimised, LAMP assays tested 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. LAMP primer sets bapA2.1 and orgA1 (Figure 4.9) recognised all *Salmonella* serovars tested. OrgA2 recognised all serovars accept *S.* Mbandaka

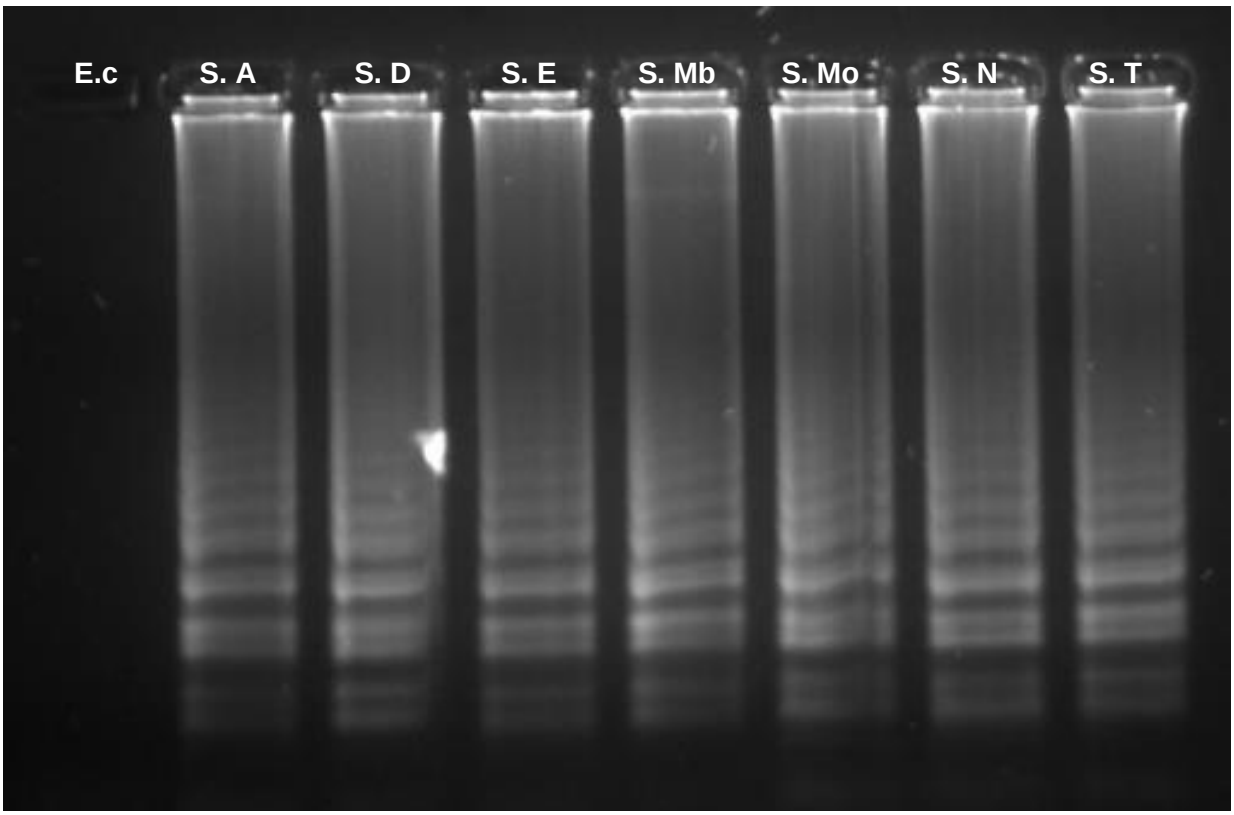

Figure 4.9: Agarose gel showing products of orgA1 LAMP assay amplified for 30 minutes at 65°C targeting multiple *Salmonella* serovars. Negative control assays, containing no template DNA showed no product.

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

4.2. Generating an immunoassay on the Vantix Reader 2 to detect pan-*Salmonella* antigen through calf scour results

To determine the binding activity of a selection of commercial antibodies targeting pan-*Salmonella*, ELISA assay was generated and optimised. Error bars represent the standard deviation within the data set and, unless otherwise stated, all graphs show the signal generated by *Salmonella* serovars minus the signal generated by the *E. coli* negative control. Direct ELISA assays were used to confirm that the commercial antibodies recognised various *Salmonella* strains specifically. Figure 4.10 shows that all three antibodies produce a greater colorimetric response for the *Salmonella* strains than for *E. coli*. It can also be observed that there is a large deviation between data sets.

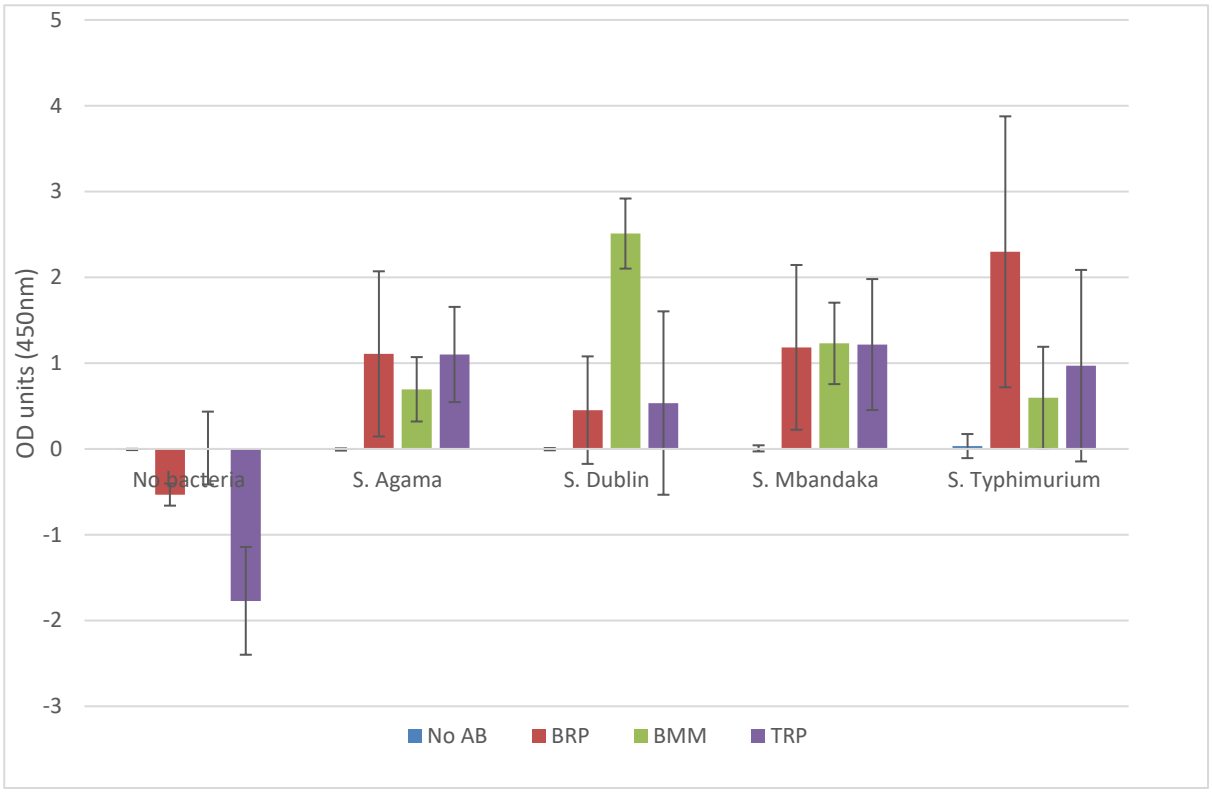

Figure 4.10: Detection of different *Salmonella* serovars by a panel of antibodies in a direct ELISA.

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

Additionally, ELISAs were used to optimise immunoassay steps allowing for the best differentiation between negative and positive results. Varying blocking solution concentration, 0.1% skimmed milk (w/v) showed best differentiation compared to 1% & 5%. When washing ELISA plates between reaction steps, a greater differentiation was seen when washing with a multichannel pipette, however less deviation between data sets, thus an increase in repeatability, was seen when washing with a

wash bottle. A greater colorimetric response was seen in ELISA assays that have been incubated at 37°C than when incubated at 4°C, however a 4°C there was less deviation between results.

For initial adaption of the optimised immunoassay to biosensor using the Vantix system, the original Vantix System (VR1) was used. A potentiometric response for *S*. Dublin and *S.* Mbandaka greater than that of the negative controls was seen when an overall incubation time of 6 hours down to 2.5 hours.

Potentiometric immunoassays on the VR2 were completed as described in section 3.9.3. 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. Further optimisation of the immunoassay occurred to increase potentiometric signal response and to increase differentiation between negative and positive results. An increase in A99H monoclonal antibody concentration from 1:500 to 1:100 resulted in better differentiation as well as an increased potentiometric signal from *S.* Dublin and *S.* Mbandaka. Overall incubation times of 2hr, 1.75hr, 1.25hr, and 1hr were tested: in general, a reduction of reagent incubation time increased the differentiation between the potentiometric signals produced by the control and test samples. However, 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 4.11).

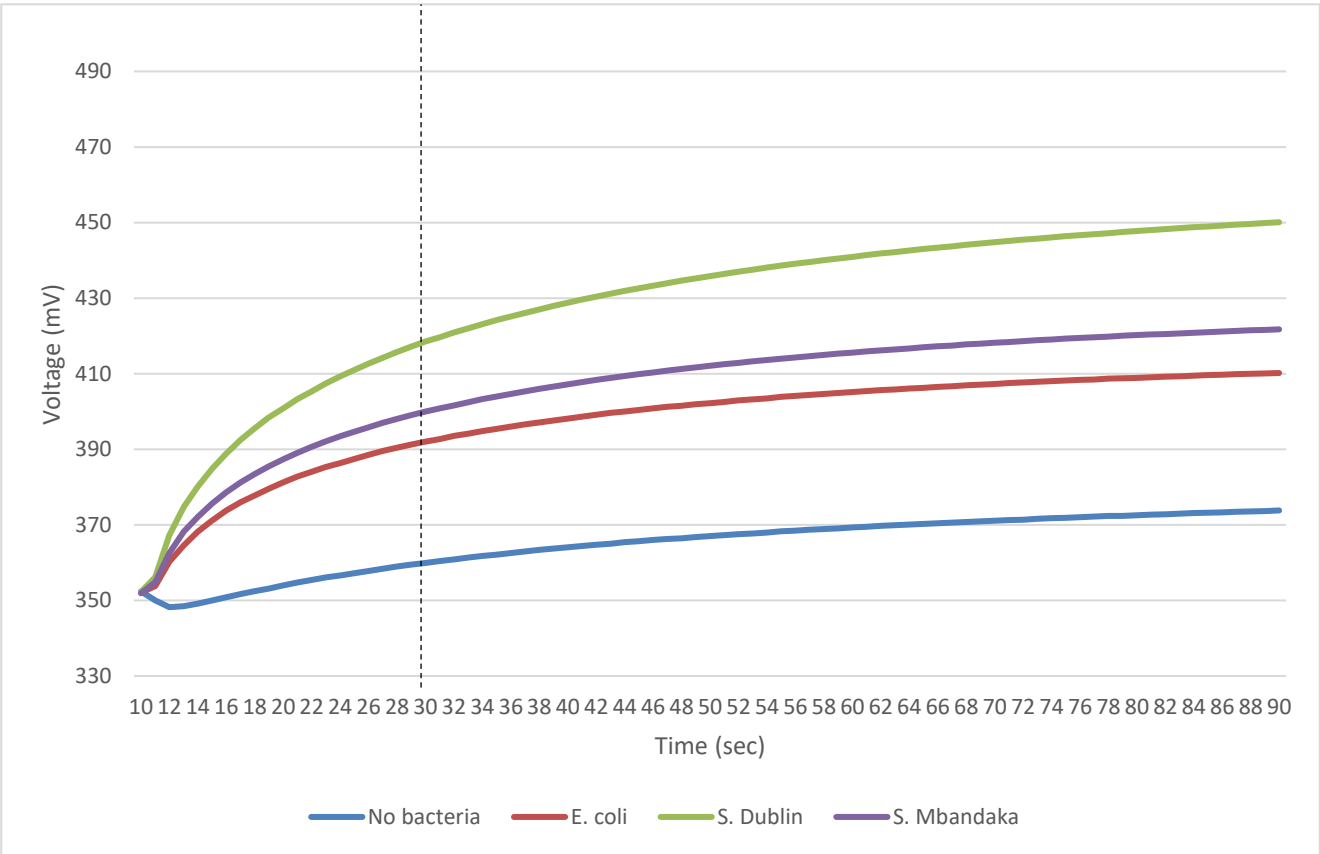

Figure 4.11: Average of Vantix Sandwich assays run on the VR2, with A99H at 1:100 dilution, using 1.25hr overall incubation time. Bacteria was standardised at 0.5 OD units
Key: dashed black line = 30 second time point

Using the 1.25hr incubation time, the specificity of the optimised potentiometric immunoassay to various *Salmonella* serovars was completed. Overall a clear differentiation between *Salmonella* serovars and the negative controls was seen at both concentrations of bacteria, this is most apparent at 30 seconds (Figure 4.12 and 4.13). *S.* Typhimurium and *S.* Agama showed the strongest potentiometric response across both concentrations of bacteria (Figure 4.12 and 4.13). At 0.25 OD units *S.* Montevideo and *S.* Newport produced a similar voltage to that of *S.* Dublin over the 90 seconds of reading (Figure 4.12). At 0.5 OD units, *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 4.13).

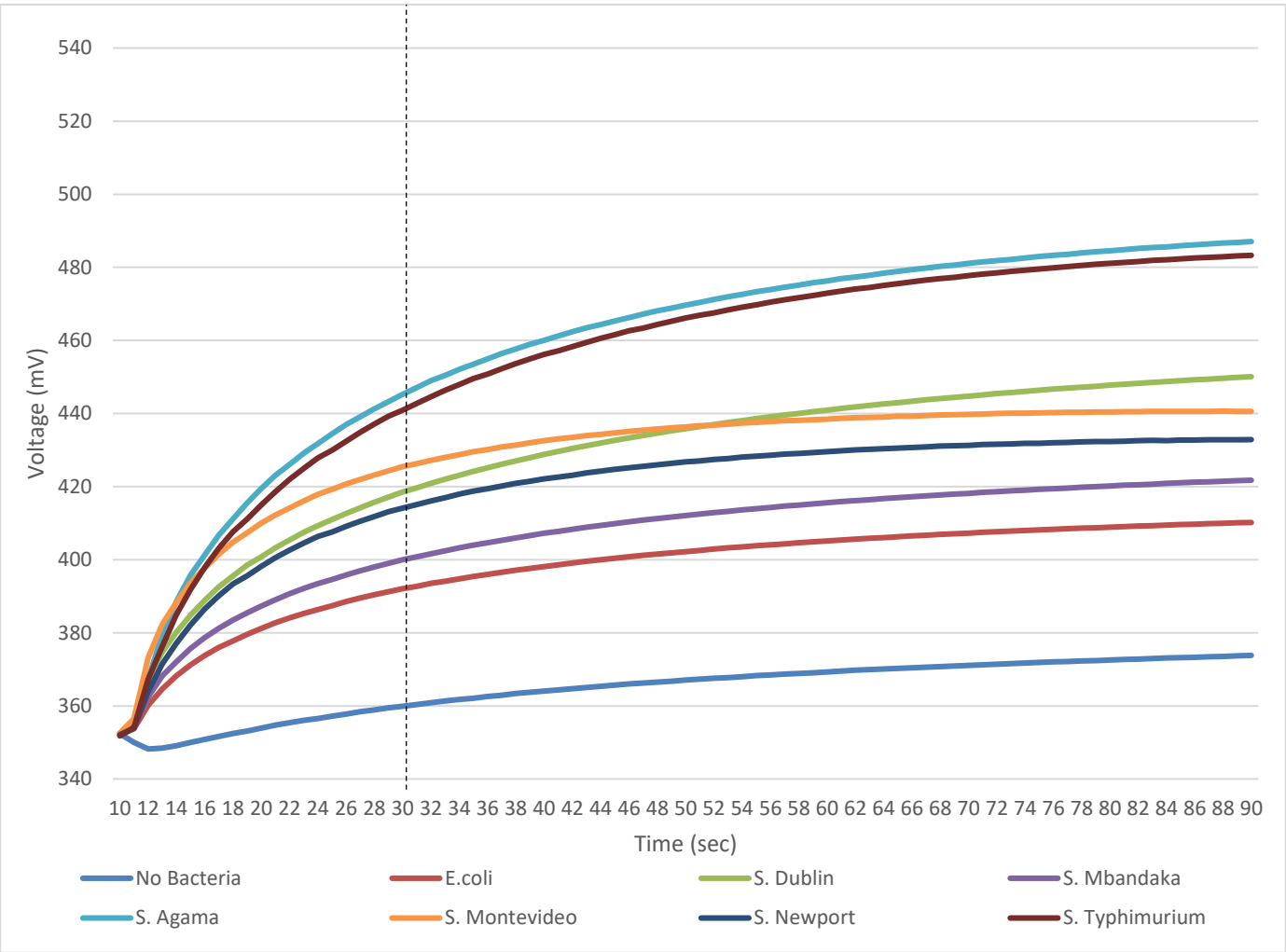

Figure X12: An average of Vantix Sandwich assays done on the VR2 testing different *Salmonella* serovars at 1.25hr overall incubation time, with bacteria standardised to 0.25 OD units.
Key: Black line = 30 second time point

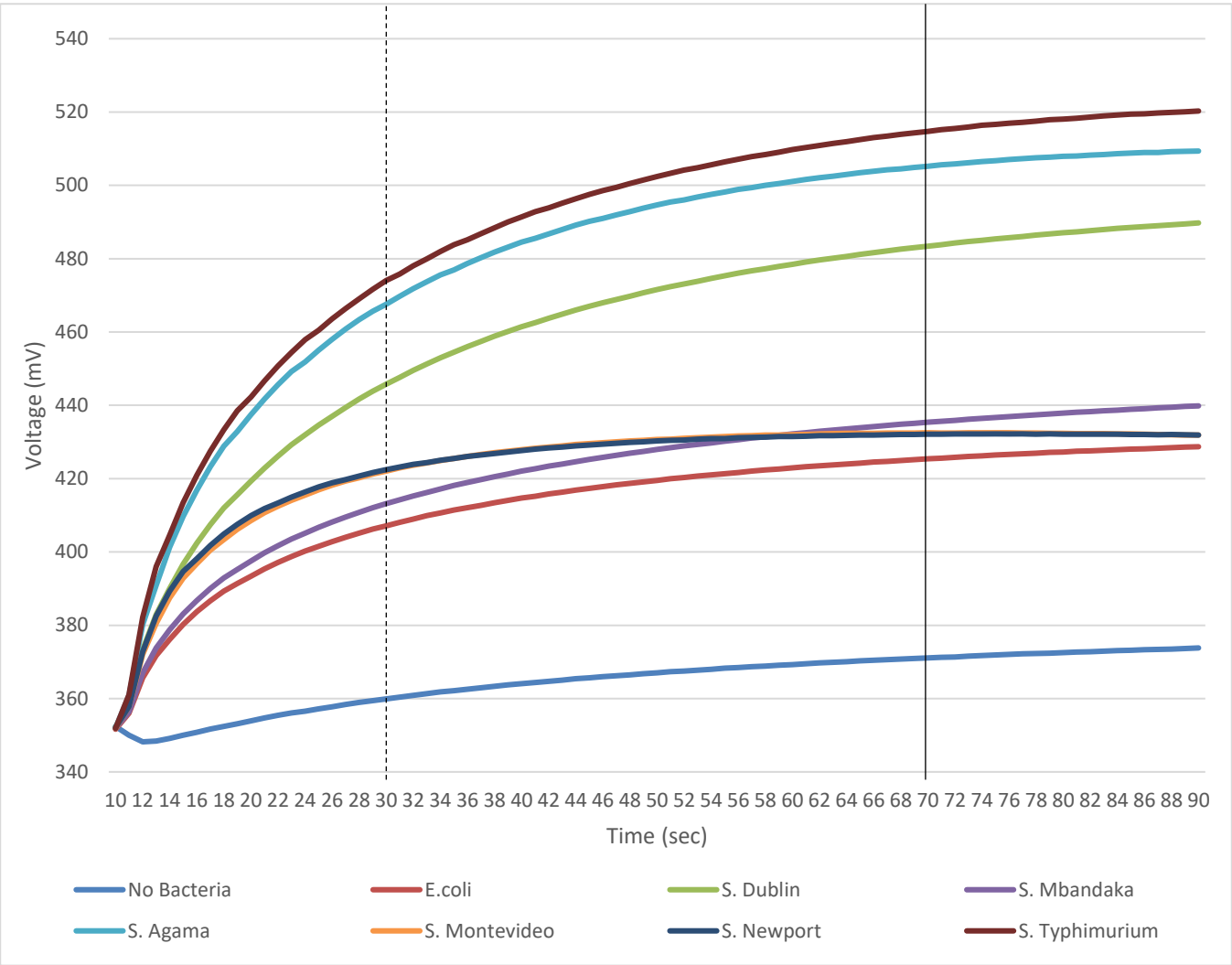

Figure X13: An average of Vantix Sandwich assays done on the VR2 testing different *Salmonella* serovars at 1.25hr overall incubation time, with bacteria standardised to 0.5 OD units.
Key: Dashed black line = 30 second time point, black line = 70 second time point

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. *Salmonella* negative scour was spiked with known quantities of *Salmonella* bacteria for testing. Bacteria was standardised to 1 OD units, then diluted 1:2 to 0.06 OD units. 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.

Overall when read through calf scour *S.* Dublin produced a muted response when compared with the Sandwich Vantix assay results, as expected. However, at 30 seconds *S*. Dublin produced a

stronger potentiometric response than the controls, except at the lowest concentration tested, 0.06 OD units.

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 1/10 dilutions of 1 – 0.25 OD units (Figure 4.14), than seen through undiluted scour. At 1/10 dilution of 0.125 OD units *S.* Dublin did not produce a higher potentiometric response than *E. coli*.

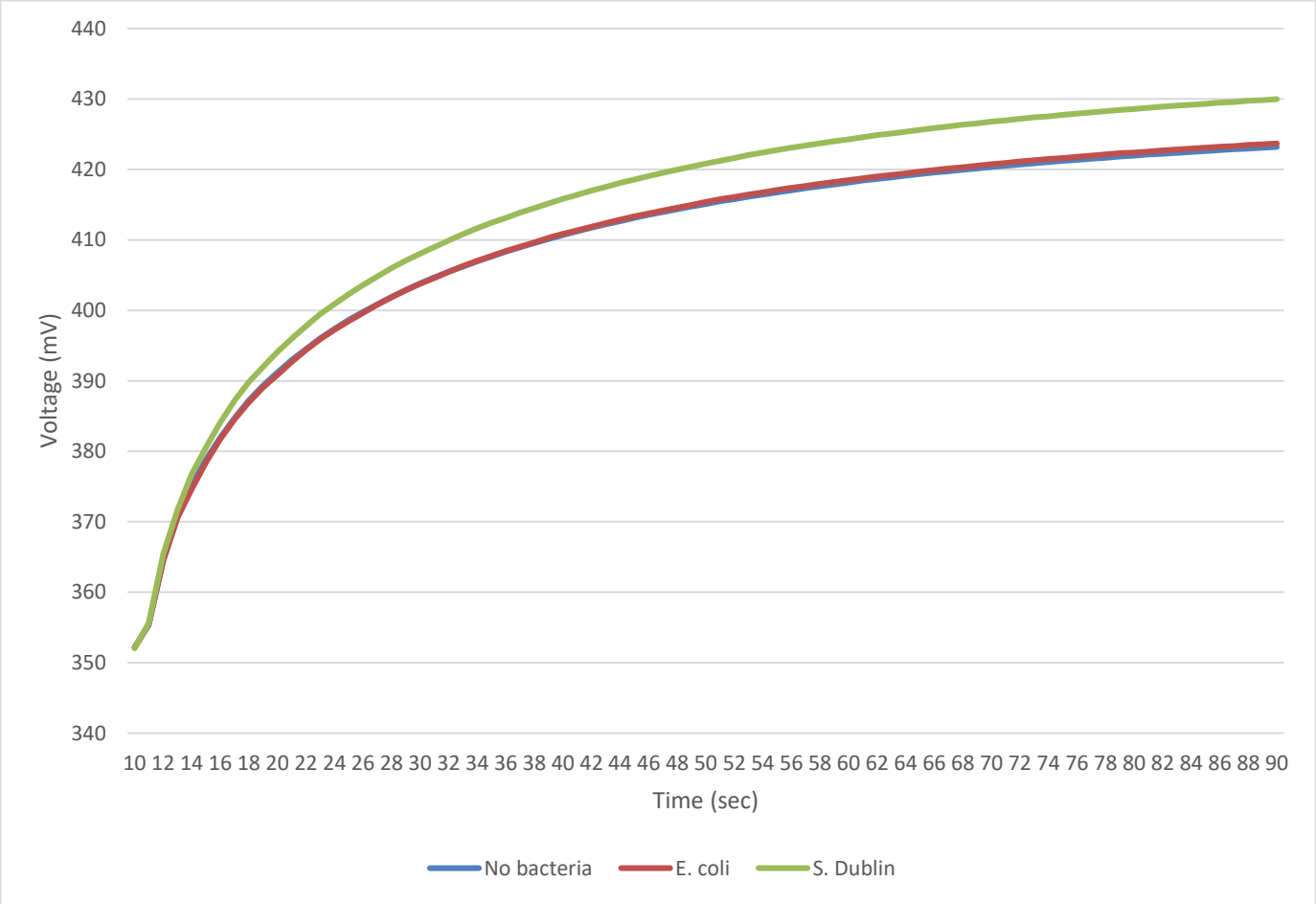

Figure 4.14: Average of Vantix sandwich assays through a 1:2 dilution of calf scour. Bacteria was diluted 1:10, from an OD of 0.5, in scour.

5. 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 (Table 4.1). 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 (Figure 4.1), however 5 primer sets recognised at least four of the six *Salmonella* serovars tested (Table 4.2). 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* (Table 4.3). 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 (Figure 4.2). 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 (Figure 4.3). 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. Whilst success was seen with Nile Blue A (Figure 4.4) and propidium iodide (Figure 4.5) 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 (Figures 4.6 & 4.7). Due to this success, SYBR Green I was screened out, despite working well, due to the expense of the dye compared to 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 (Table 4.4) compared to SYBR safe (Table 4.5) 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, (Tables 4.4 & 4.5) 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 (Table 4.6). 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, primer sets bapA2.1 and orgA1 (Figure 4.9) 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 (Figure 4.10). 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 (Figure 4.11). 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 (Figures 4.12 & 4.13). 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 $4.07x10^6$ 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 (Figure 4.14). The signal produced in the presence of *S*. Dublin in diluted scour was greater than that seen through undiluted scour.

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. The potential for cheap, easy mass production and the ability to simply adapt established ELISA techniques, 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.

6. Industry messages

To reduce illnesses associated with food products, a multifaceted approach from farm to table is needed. *Salmonella* sp. causes gastroenteritis in humans, impacting public health. S*almonella* sp. have a high impact on economics and animal welfare, causing a high level of sickness in infected cattle, and a high death rate amongst infected calves. As asymptomatic *S*. Dublin carriers can excrete bacteria in milk and faeces, 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. A rapid and inexpensive diagnostic kit would be a useful in this situation ensuring *Salmonella* infections are controlled.

Despite diarrhoea a common symptom of salmonellosis, scour can also be caused by viruses, such as BVD, and parasites, such as lung worm. Salmonellosis can kill calves within 48 hours, which with current methods is quicker than a diagnosis. 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, that governments worldwide are acting upon. 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, already the emergence of MDR *Salmonella* strains are beginning to limit treatment options within cattle herds. 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.

To aid this, two rapid detection methods have been developed to target *Salmonella* sp.;

- A potentiometric immunoassay for the detection of *Salmonella* serovars developed on the Vantix Reader 2 (VR2) is rapid, completed and read in under an hour, and is robust through calf scour. The biosensor immunoassay has the potential for cheap, easy mass production, as well as the ability to simply adapt established ELISA techniques utilising commercially available antibodies, the VR2 shows great promise as a rapid detection system that could easily be immediately utilised.
    - The VR2 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.

- A fluorometric LAMP assay for the detection of *Salmonella* sp. that is quick and simple, with visible results generated in 40 minutes. Future research is needed to finalise protocol and ensure robustness through scour, LAMP shows promise as a detection method for *Salmonella* serovars on site, pen-side to infected cattle due to high specificity and sensitivity.
    - Pan-*Salmonella* fluorometric LAMP assay would be well 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.

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.

Additionally, use of these diagnostics 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.

Controlling the spread of *Salmonella* sp. would protect herds, increasing animal welfare and reducing the economic impacts of salmonellosis. The potential for food contamination would be reduced, preserving public health. By targeting treatment with quick reliable diagnostics, cattle can receive the correct treatment for the correct disease, safeguarding antimicrobials and staying ahead of government legislation. 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 being simple to use, with easily interpretable results.

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