# Point of management (POM) assays for early detection of *Escherichia coli* and *Actinobacillus pleuropneumoniae* in effluent.

## Final Report

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We would also like to acknowledge our PhD student, who worked on the best extraction method. Her paper is attached as an appendix to this report.

## Executive Summary

The recent development in response to the SARS-COVID-19 pandemic of a monitoring system for a respiratory disease by screening sewage has raised the possibility of a similar approach to monitor pig respiratory pathogens. Previous evidence of several pathogenic bacteria isolated from effluent via culturing method with dilution and selected media by Chinivasagam et al. (2004), suggests that piggery effluent could also be used as a point of management (POM) diagnostic tool. This monitoring could help with early detection and improved control of disease outbreaks that could cause significant economic losses and reduced animal welfare. This POM approach and better control of infectious diseases would also reduce the need for antimicrobials, improving the sustainability of intensive pig production.

This project consisted of three steps. The first step in this project was to confirm, using standard laboratory-based technologies such as polymerase chain reaction (PCR) and a developed loop-mediated isothermal amplification (LAMP) assay, that a common bacterium, *Campylobacter coli*, can be detected in effluent. Further, to demonstrate that with standard laboratory-based technologies, such as PCR, *Actinobacillus pleuropneumoniae* can be detected in spiked effluent. The second step was to develop a POM assay for *A. pleuropneumoniae,* and the third step was to develop a POM assay for *Escherichia coli*.

**Step 1 A: Detection of *Campylobacter coli* in effluent**

Detection of *Campylobacter coli* in effluent samples was undertaken using a LAMP assay previously developed and validated by Mason et al. (2019). Effluent was collected from three commercial piggery operations in southeast Queensland, and the presence of *C. coli* in the samples was first verified through culture-based analysis.

Method: For molecular analysis, DNA was extracted from 15 mL effluent samples using several different approaches. These included two commercial extraction kits (QiaAmp Power Faecal Kit and NucleoSpin Soil Kit) as well as a laboratory-developed protocol designed in this study that relied on chemical and heat-based cell lysis.

Results: The extracted DNA was subsequently screened for *C. coli* using both conventional PCR and LAMP. These assays were based on previously published protocols by Wang et al. (2002) and Mason et al., (2019) with minor adjustments. Specifically, Platinum II Taq Hot-Start DNA Polymerase was used in the conventional PCR reactions, while Bst 3.0 DNA polymerase was used for the LAMP assay.

Although the samples were confirmed to contain *C. coli* by culture, neither the conventional PCR nor the LAMP assay initially produced positive results, regardless of the DNA extraction approach used. When the conventional PCR was repeated using Platinum II Taq Hot-Start DNA Polymerase, *C. coli* was detected in four samples where DNA had been extracted using the laboratory-developed method and in one sample processed with the QiaAmp Power Faecal Kit.

Following further optimisation of the LAMP assay with Bst 3.0 DNA polymerase, all samples tested positive for the presence of *C. coli*, independent of the DNA extraction method employed.

Summary: This study demonstrated that testing effluent with a LAMP assay can be used as a point of management (POM) approach to detect pathogens in pig herds. The enhanced detection observed in this study is likely attributable to the use of Platinum II Taq Hot-Start DNA Polymerase and Bst 3.0

DNA polymerase, which appear to be more tolerant of inhibitory substances present in effluent compared with the polymerases used in the originally published versions of these assays.

**Step 1 B: Detection of *A. pleuropneumoniae* in spiked effluent through PCR**

For the second part of step 1, effluent was spiked with a 10-fold dilution of *A. pleuropneumoniae*, and DNA was extracted from the effluent using the developed BentoLab DNA extraction method, with and without the Zymo column, and with the NucleoSpin extraction kit. A conventional PCR (Turni et al., 2014) was used to test the extracted DNA.

Results: The conventional PCR only reached a sensitivity of $10^6$ cfu/ml with the BentoLab plus Zymo column extraction and with the Nucleo Spin soil Kit. This low sensitivity was again attributed to the inhibitors in the effluent. After changing the polymerase of the conventional PCR, the detection limit was $10^2$ cfu/ml.

Summary: This demonstrates that *A. pleuropneumoniae* is detectable in effluent.

**Step 2: Optimisation of a LAMP assay for *A. pleuropneumoniae* using previously developed primers.**

Primers for *A. pleuropneumoniae* from a previously developed LAMP assay (Yang et al., 2009) were used. The LAMP assay developed by Yang et al. (2009) was not sensitive enough to detect *A. pleuropneumoniae* in effluent and lacked a working colourimetric detection method.

Method: After optimisation by replacing the polymerase and buffers with the WarmStart Colorimetric LAMP 2 x Master Mix, the sensitivity and specificity of the assay were reassessed.

Results: The sensitivity for *A. pleuropneumoniae* was 2.88 x $10^3$ cfu/ml (2.88 cfu/µl) and the assay displayed 100% specificity.

**Step 3: Development of a LAMP assay for *E. coli*.**

The WarmStart Colorimetric LAMP 2 x Master Mix was used, and 5 newly designed primer sets were initially evaluated for optimisation. These primers were evaluated for the detection of known STb positive and negative isolates of *E. coli*. Once one set was detected that amplified the correct isolates, we then optimised this set.

Method: Once the assay was established, it was tested using known STb-positive and STb-negative *E. coli* isolates. After identifying a primer set that correctly amplified the target isolates, this set was selected for further optimisation. To further improve sensitivity and reduce amplification time, guanidine chloride was incorporated into the reaction. After determining the optimal amplification time and guanidine chloride concentration, the sensitivity and specificity of the assay were evaluated.

Results: The sensitivity for *E. coli* was 2.65 x $10^2$ cfu/ml (0.265 cfu/µl), and the assay displayed 100% specificity.

Summary of steps 2 and 3: The objectives of developing qualitative POM assays that can detect respiratory and enteric bacterial pathogens in effluent were achieved. Both assays displayed a sensitivity similar to real-time PCR and were both 100% specific.

This can serve as a platform for other bacterial pathogens, and the lessons learned on optimising the LAMP assay can be applied to developing other LAMP tests.

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## 2 Background to Research

During the COVID-19 pandemic, wastewater surveillance was used to track how widely the virus spread within communities. Piggery effluent, which contains faeces, urine, respiratory secretions, leftover feed and other materials diluted in water, is released into the environment as a mixed waste stream (Rate, 1997). Because this mixture has previously been shown to contain various pathogenic bacteria (Chinivasagam et al., 2004), it may offer a practical way to assess pathogen levels within a pig herd at a single point in time. Such monitoring could support early disease detection and improve outbreak control, helping prevent economic losses and protecting animal welfare.

Using this point of management (POM) strategy could also reduce antimicrobial use, support sustainable pig production and lower the risk of antimicrobial resistance spreading into the food chain or environment. Loop-mediated isothermal amplification (LAMP), a nucleic acid-based method, is widely used for POM diagnostics due to its sensitivity, reliability, low cost, and rapidity. This study, therefore, aimed to develop a proof-of-concept LAMP-based effluent testing approach to determine whether it can detect pathogens at the shed or herd level. Given the nature of effluent and the practicality of LAMP testing, it was proposed that LAMP could successfully identify common pathogens in pig effluent for POM diagnostic purposes.

Currently, a common practice in pig production systems to prevent bacterial disease outbreaks is the use of antimicrobial agents, as no on-farm monitoring is available. Farmers rely on sending samples to diagnostic labs, which is done when the disease outbreak is in full swing. To avoid this, monitoring tools are needed to inform the farmer when bacterial levels are elevated, and intervention programs are needed. We aimed to demonstrate that LAMP assays would be the ideal platform for on-farm monitoring, as they need no complicated laboratory methods or equipment. The idea of this project was to start by demonstrating the concept using a developed LAMP assay for *Campylobacter coli* (Mason et al., 2019). The extracted DNA from bacteria in the effluent was tested for the presence of *C. coli* using both conventional PCR and the LAMP assay, as originally reported by Wang et al. (2002) and Mason et al. (2019), respectively. We had to optimise both assays to improve sensitivity.

### *1.1 Campylobacter coli*

*Campylobacter coli* is a common bacterial inhabitant of the gastrointestinal tract of pigs and is widely detected on pig farms worldwide. Studies have shown that pigs frequently carry *C. coli* asymptomatically, with prevalence levels on farms reported at 60–90%, indicating that swine are a major reservoir of this organism (Boes et al. 2005; Harvey et al., 1999). In Australia, a nationwide survey of pig herds, sampling 300 pigs, reported a *C. coli* carriage rate of approximately 72.7%, demonstrating that the bacterium is highly prevalent in Australian commercial pig production systems (Owiredu et al., 2023). Despite its high prevalence, *C. coli* generally causes little or no clinical disease in pigs and is considered a commensal organism of the intestinal tract; however, infected animals can shed large numbers of bacteria in faeces, facilitating environmental contamination and transmission within farms and along the food chain (Harvey et al., 1998). This widespread colonisation in pig populations is significant from a public health perspective because *Campylobacter* species are among the leading causes of bacterial gastroenteritis in humans, and livestock reservoirs contribute to contamination of meat products and the broader food production environment (McLure et al., 2023).

This high prevalence in pig herds and the high likelihood of environmental spread made this bacterium an ideal candidate to test whether the LAMP assay can detect bacterial species in effluent.

### 1.2 *Actinobaccilus pleuropneumoniae*

The bacterium *A. pleuropneumoniae* is classed into 19 serovars with each of them producing different combination of the pore-forming repeat-in-toxin family members, the Apx toxins (I to IV) (Sassu et al., 2018). Pathogenicity depends on the toxins present, with each toxin varying in its degree of cytotoxicity and haemolytic activity. ApxI is strongly haemolytic and strongly cytotoxic and is produced by serovars 1, 5, 9, 10, 11, 14 and 16. ApxII is weakly haemolytic and moderately cytotoxic and occurs in all serovars except 3, 10 and 14. ApxIII is non-haemolytic but strongly cytotoxic and is expressed by serovars 2, 3, 4, 6, 8 and 15 (Stringer et al., 2021) (Stringer et al., 2021). ApxIV is produced by all serovars *in vivo* and appears to enhance pathogenicity by its antimicrobial activity, thereby facilitating invasion (Slivenecka et al., 2025). As all *A. pleuropneumoniae* isolates produce this toxin, which is not found in other *Actinobacillus* species (Frey, 2003), it is the most commonly used target for diagnostic tests.

*A. pleuropneumoniae* is transmitted by direct contact and through mucus and aerosols from one pig to another, making it very contagious and easily spread throughout the herd (Niven and Levesque, 1988; Assavacheep and Rycrog, 2013). It can remain airborne and exhibits greater stability in water and nasal discharges, remaining viable for over 10 weeks in water and nearly 5 days in mucus. The bacterium is viable in water, making it a potential reservoir and a vehicle for transmission (Assavacheep and Rycrog 2013).

For on-farm detection of *A. pleuropneumoniae,* primers were adapted from an established LAMP assay that was relatively insensitive (Yang, 2009). We optimised the LAMP assay to increase the sensitivity without losing specificity.

### 1.3 *Escherichia coli*

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common pathogens causing post weaning diarrhoea in pigs. In 2015, a PhD student at the time, Dr Rousset Palou, examined 89 *E. coli* isolates obtained from piglet faeces, small intestine and colon samples collected in weaning sheds on Australian farms, which were selected and tested for virulence genes. Of these 11 did not have the virulence genes and were discarded as ETEC strains (Table 1.). The most common fimbrial gene was F18, found in 47% of isolates, followed by F4 at 30%. The heat-stable enterotoxin type b (STb) gene was present in 63% of the isolates, the highest frequency among the toxin genes. The virulence gene (VG) profile F4:STa:STb:LT and F18:STb were the most common with a 18% and 17%, respectively. This finding agreed with previous reports from Australia (Van Breda et al., 2017, Do et al., 2005, Smith et al., 2010) and other countries (Chen et al., 2004, Zhang et al., 2007, Frydendahl, 2002), where isolates carrying F4, F18 and STb were presented in high frequencies, suggesting similar VG profiles to the ones found in this experiment. As the STb toxin was the most commonly detected virulence gene, we selected it for ETEC detection using the LAMP assay.

The benefit of this project is the creation of monitoring opportunities that allow the farmer to make informed decisions and, in turn, reduce antibiotic use. If we want to reduce antibiotic use, we need to bring knowledge to the farm so that the farmer and veterinarian can make informed decisions in real time, rather than waiting for an outbreak and then reacting to it.

Table 1. Quantity of isolates in each virulence gene profile after 3 different multiplex PCRs in 89 field strains of *E. coli* collected from pig farms with cases of weaning diarrhoea in Victoria (17), New South Wales (47), Queensland (20), South Australia (3) and Western Australia (2) (Rousset Palou, 2019).

| Fimbrial gene | No. isolates | Toxin genes | | | | | | Additional genes | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sta | STb | Sta:STb | STb:LT | Sta:STb:LT | Negative | *eaeA* | *stx*$_2$ | *hlyA*:*eaeA* | *stx*$_2$:Sta | *stx*$_2$:STb:LT |
| F4 | 22 | 0 | 0 | 1 | 5 | 16 | 0 | 0 | 0 | 0 | 0 | 0 |
| F5 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F18 | 38 | 0 | 15 | 2 | 1 | 6 | 4 | 0 | 8 | 0 | 2 | 0 |
| F4:F5 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| F4:F6 | 4 | 0 | 0 | 0 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| F4:F18 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| F6:F18 | 3 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| Negative | 18 | 0 | 1 | 0 | 2 | 2 | 11 | 1 | 0 | 1 | 0 | 0 |

# 3 Objectives of the Research Project

1. Produce a proof-of-concept Point of Management (POM) assay validated for use on piggery effluent. This proof-of-concept assay was based on an existing validated assay for *Campylobacter coli* and chicken carcase rinse and required only validation on the new effluent matrix.
2. Develop a LAMP assay for *Actinobacillus pleuropneumoniae* that can detect the bacterium in effluent. The LAMP assay was validated by standard molecular-based laboratory techniques.
3. Develop a LAMP assay that can detect *Escherichia coli* in effluent. The LAMP assay was validated by standard molecular-based laboratory techniques.

# 4 Introductory Technical Information

## 4.1 *LAMP assay*

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification technique that operates at a constant temperature and is known for its high specificity and efficiency (Tsugunori et al., 2000). The method uses a set of specially designed primers that recognise multiple regions of the target DNA sequence, allowing precise amplification. LAMP relies on a loop-based mechanism in which DNA synthesis proceeds continuously while the reaction temperature remains optimal for the Bst DNA polymerase (Figure 1). DNA polymerases used in LAMP are specifically adapted to function under isothermal conditions and possess strong strand-displacement activity, enabling amplification without thermal cycling. The assay typically employs four main primers along with two additional loop primers that accelerate the reaction and increase amplification speed. Compared with other nucleic acid amplification methods, LAMP is often more sensitive, particularly when detecting low concentrations of DNA. This increased sensitivity is largely due to its exponential amplification mechanism and the strand-displacement activity of Bst polymerase, which eliminates the denaturation step required in conventional PCR. The tubes do not need to be opened, and no electrophoresis is needed as the amplification products can be detected by a simple colour change with high sensitivity (less than 10 copies) in around 30 minutes (Figure 2) (Tanner et al., 2015) (Asadi and Mollasalehi, 2021).

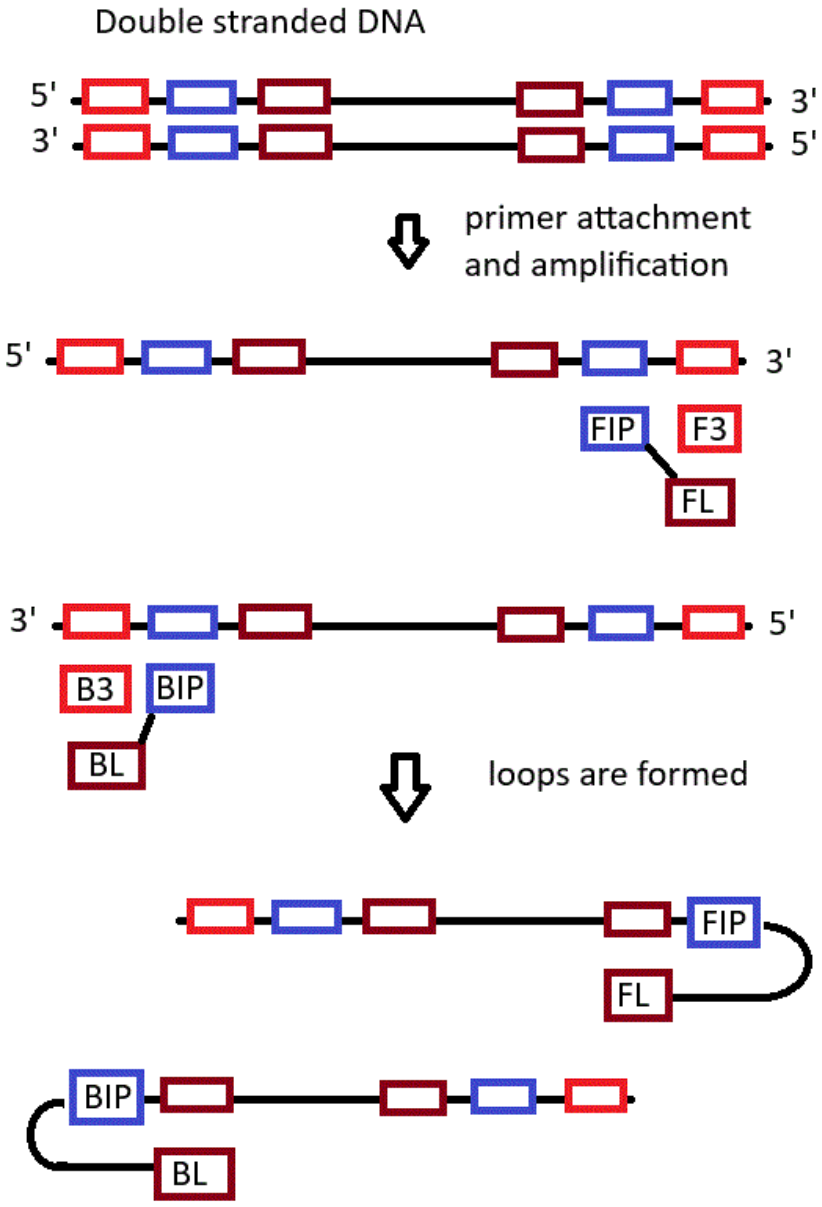



Figure 1. LAMP amplification depicted. Primers are the labelled boxes. Unlabelled boxes are the binding sites of the primers on the DNA strand. The primers bind, and both FIP and F3 initiate amplification. If loop primers are used, they will form a loop in the amplified strand, which will then be amplified.

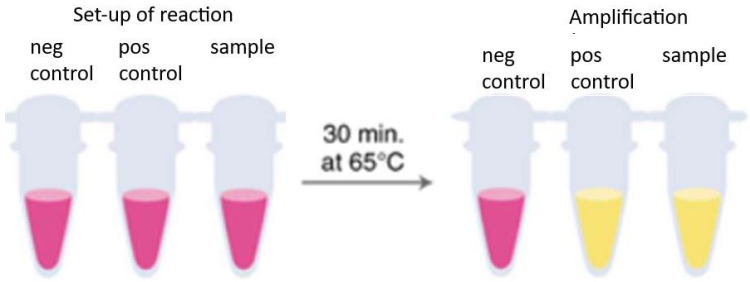



Figure 2. Colourimetric LAMP assay depicting the colour change that occurs during amplification. The reaction changes from red to yellow when amplification occurs.

## 5 Research Methodology

Three different assays were developed and optimised. As this is about developing methods, we will describe only the optimisation we performed, then present the final assay in the results, including the optimisation outcome and the test we conducted. Several methods have been used to develop a LAMP assay. To establish whether the colour reaction actually shows amplification, we ran a species-specific PCR (either conventional or quantitative) on the DNA extracts and performed electrophoresis of the LAMP product to confirm that the colour change was indeed due to amplification. We then tested the LAMP assay's sensitivity by spiking the effluent with a known positive sample. The next step was to test the specificity. This was done by spiking the effluent with related species.

### *5.1 General methods for laboratory testing*

#### *5.1.1 Spiking*

Sensitivity was assessed by spiking the effluent with known bacterial concentrations. The overnight bacterial culture was harvested and suspended in 6 ml of PBS with an estimated concentration of $6\times10^8$ CFU/ml, based on turbidity matching the McFarland standard 2.0. A serial dilution was prepared in PBS from this original concentration by adding 1ml of the initial bacterial mixture into 9 ml PBS. Dilutions were counted by plating 100 µl onto TM/SN agar (for *A. pleuropneumoniae*) or blood agar (*E. coli*), and incubating for 24 hours at 37°C.

For spiking of effluent, 1 ml of each dilution was then added to 14 ml of effluent. These spiked dilutions were then extracted via the magnetic bead method. Each extracted DNA sample from each spiked dilution was tested with the LAMP assay to assess assay sensitivity.

#### *5.1.2 Related bacteria testing*

To ensure that the LAMP reaction does not cross-react with other bacterial species that are pathogenic in piggeries, we tested the assay against the bacterial species listed in Table 2. We used two extractions to assess whether related species yielded false positives: first, by spiking effluent with related bacterial species and extracting DNA via the magnetic bead method. Second, by suspending the bacterial colony in water and crude extraction of the DNA via the boiling method without the use

of effluent. The spiking was performed as described above with 1 ml of $10^7$ cfu/ml of each bacterial species mixed with 14 ml of effluent. The crude DNA extraction from *Streptococcus suis* was performed using the Qiagen Blood and Tissue Kit.

Table 2. Related species tested for specificity of the LAMP assay

| Species | Code |
|---|---|
| *Glaesserella (Haemophilus) parasuis* | CCUG 3712 |
| *Actinobacillus indolicus* | CCUG39029 |
| *Actinobacillus minor* | CCUG38923 |
| *Actinobacillus porcinus* | CCUG38924 |
| *Actinobacillus suis* | CCUG11624 |
| *Bordetella bronchiseptica* | BR467 |
| *Pasteurella multocida* | NCTC10322 |
| *Pasteurella species B* | SSIP683 |
| *Streptococcus suis* | CCUG7984 |
| *Escherichia coli* | AICC25922 |
| *Erysipelothrix rhussiopathiae* | CCUG221 |
| *Salmonella enterica serovar Typhimurium* | BR224 |
| *Campylobacter coli* | C5015 |
| *Glaesserella australis* | HS4635 |
| *E. coli* with STb gene | BR1602 |
| *E. coli* without STb gene | BR1616 |

### *5.1.3 DNA extraction from pure culture of related species*

A 1 µl loopful of culture was harvested into 200 µl of $H_2O$, vortexed, then heated at 98°C for 5 minutes and cooled on ice for 5 minutes. These steps of heating and cooling were repeated, and then the solution was centrifuged. The supernatant is used for PCR or LAMP assay.

### *5.1.4 Magnetic Bead DNA extraction method from effluent*

The spiked tube of effluent was used to extract DNA for the development of the LAMP assays. The method described here is similar to the approach later referred to as the Bento Lab method for the on-farm DNA extraction. In this case, however, it was conducted under laboratory conditions with bigger volume of effluent samples for assay validation. The solution was centrifuged at 1000 × *g* for 5 minutes to settle the heavier solids. The supernatant was centrifuged at 4550 × *g* for 30 minutes. After discarding the supernatant, 500 µl of lysing buffer (0.1 M EDTA, 0.1 M Tris, 0.5 M NaCl) was added to resuspend the pellet. The solution was transferred to a 2 ml tube, and 71.4 µl of 10% SDS was added. The mixture was then heated at 98°C for 20 minutes and vortexed every 5 minutes for 3 seconds. The solution was then placed on ice for 5 minutes, heated again at 98 °C for 10 minutes, and then incubated on ice for 5 minutes. The solution was then centrifuged at 8000 × g for 10 minutes, and the supernatant was transferred to a 2 ml LoBind tube (Eppendorf®, Hamburg, Germany). AMPure XP reagent (Beckman Coulter – Brea, CA - USA) was added at a 1:1 ratio, with a maximum of 400 µl being added to each sample.

Samples were incubated at 20˚C for 15 minutes with shaking at 300 rpm. This was followed by placing the tubes on an Invitrogen™ (Waltham, Massachusetts, United States) DynaMag™-2 magnetic rack to

remove the liquid. The beads were washed twice with 700 µl of freshly prepared 70% ethanol. Following this, the tubes were left with the lids open to evaporate the ethanol for 10 minutes. After the tubes were removed from the magnetic rack, 100 µl of Milli-Q® water was added, and the samples were incubated at 37˚C for 15 minutes. After placing the tubes onto the magnetic rack again, the eluted DNA was transferred to a new low-binding tube.

#### *5.1.5 Electrophoresis*

The LAMP products are run on a 1% agarose gel in TAE buffer, stained with ethidium bromide, run for 1 hour at 100 V and visualised under UV light to confirm that the colour changes observed were true amplifications.

Quantitative PCR (qPCR) were also used to confirm amplification and compare sensitivities.

### 5.2 Campylobacter coli *methods*

Effluent samples were collected from two commercial piggeries identified as Farm I and II. The presence of *C. coli* was initially tested by culture in the effluent.

DNA was extracted from 15 mL of effluent by using multiple approaches, including commercial DNA extraction kits (QiaAmp Power Faecal Kit, QIAGEN, Clayton, VIC, Australia) and NucleoSpin Soil Kit (MACHEREY-NAGEL, Düren, Germany) and the Filtration and BentoLab extraction method developed in this study (using chemical and thermal lysis).

The extracted DNA was screened for the presence of *C. coli* with both a conventional PCR (Wang et al. 2002) and a LAMP assay as originally reported by (Mason et al. 2020). Minor modifications were applied in these methods, including the use of Platinum II Taq Hot-Start DNA Polymerase for the conventional PCR and Bst 3.0 DNA polymerase for the LAMP assay.

#### *5.2.1 Laboratory-based DNA extraction methods from effluent*

Two methods where developed: a filtration method and a Bento Lab method.

##### *5.2.1.1 Filtration:*

A volume of 15 ml effluent was sequentially filtered through 11 µm and 0.22 µm pore size filters. The pre-filtering step with the 11 µm was to remove the larger particle sizes. The 0.22 µm filter was used to capture the bacteria. This filter was then transferred to a 15 ml tube. Two methods were used. Either lysing buffer and proteinase K were added or just lysing buffer. This solution was then heated and cooled, and the AMPure XP magnetic beads were added to extract the DNA. The DNA was then diluted in water.

##### *5.2.1.2 BentoLab:*

This method was developed for the Bento Lab machine, a portable PCR machine designed for on-farm use (Figure 3). The principle of this method is that the effluent is distributed into small tubes. A light spin was used to remove large particles, then a hard spin to pellet the bacteria. Once the bacteria are

pelleted, the lysing buffer is added, and the mixture is then heated and cooled. The AMPure XP magnetic beads are then used to wash the DNA and elute it afterwards. A further treatment was to run the extracted DNA through a Zymo column to remove inhibitors as described below.

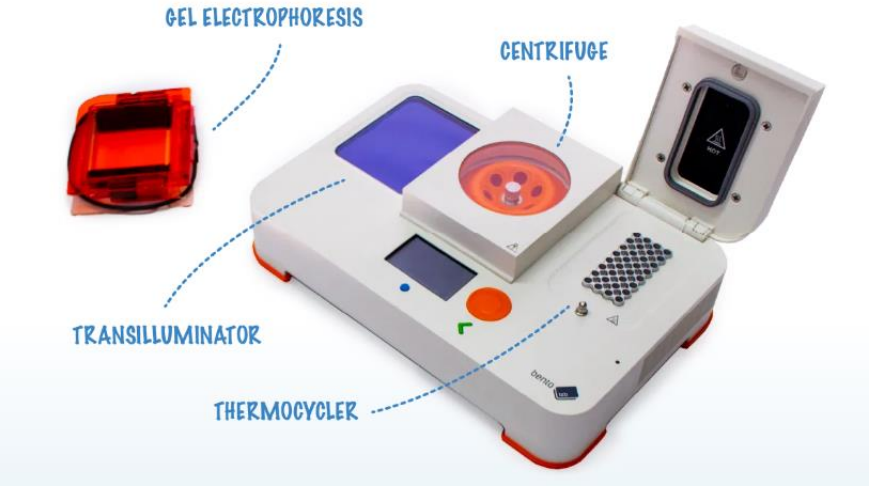



Figure 3. Bento Lab (Bento Bioworks Ltd, London, UK) sales@bento.bio

### *5.2.2 Optimisation*

Of the final DNA solution eluted from the AMPure XP magnetic beads, 75 µl was passed through the ZYMO one step PCR inhibitor column (Figure 4) and then used as the template in the conventional PCR with Platinum II Taq Hot-Start DNA Polymerase.

Figure 4. Zymo Spin Column for the purification of nucleic acids with a capacity to purify 800 µl.

## *5.3* Escherichia coli *methods*

### *5.3.1 Starting method*

In a previous study on *E. coli* from diarrheic pigs, analysis of toxin genes revealed that the *stb* gene was the most prevalent. Other studies overseas have confirmed this observation, with Moon et al. (1986) reporting that 74% of isolates from Thailand had the STb toxin, and Paiva et al. (2025) reporting that

93% of isolates in the USA had the toxin. In 2008, Dubreuil concluded that STb is responsible for colibacillosis (Dubreuil, 2008). The *stb* gene is 216 bp in length and is typically carried on plasmids. Therefore, the *stb* gene was selected as the target for primer design.

A total of five primer sets (Sets 1, 6, 20, 22 and 32) were designed using Primer Explorer V5. Of these, only Set 32 included a single loop primer, for which four variants (Loops 1-4) were designed. All primer sets were evaluated using a collection of *E. coli* isolates that either carried or lacked the *stb* gene (Table 3). The primer set demonstrating the highest specificity was selected for optimisation of the LAMP assay. This report presents only the results obtained using this primer set (Set 20) (Figure 5).

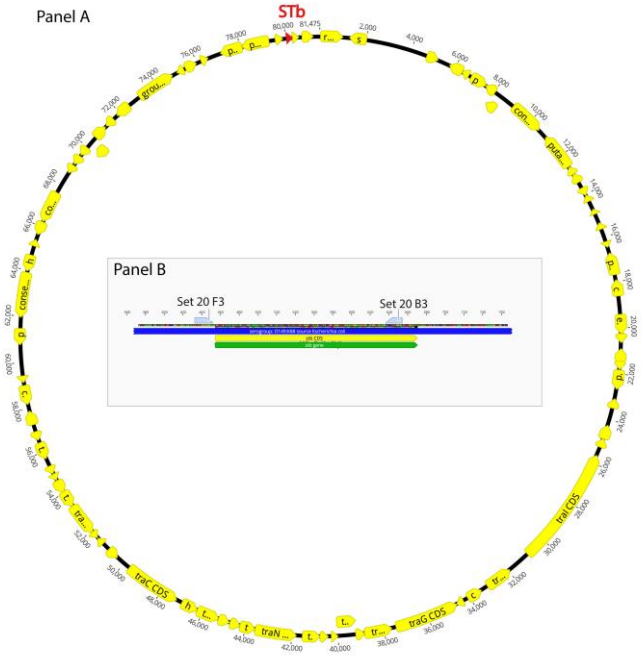



Figure 5. Schematic of *E. coli* plasmid carrying the *stb* gene. Panel A, the 81,475 bp plasmid pUMNK88 with *stb* gene highlighted in red. Panel B, the 216 bp *stb* gene with the selected B3 and F3 primers is depicted.

Table 3. List of isolates with or without the STb toxin gene. They have been tested with both conventional and quantitative PCR. The DNA was quantified on QUBIT™ (Invitrogen™, Massachusetts, USA) using the dsDNA Broad Range kit following the manufacturer's instructions.

| Isolate number | STb PCR | qPCR CT score | Qubit DNA quantification measurement |
|---|---|---|---|
| BR1602 | + | 10.5 | |
| BR 1303 | - | 0 | |
| BR 1599 | - | 0 | 22.2 |
| BR 1606 | + | 18 | 15.5 |
| BR 1601 | - | 0 | 20 |
| BR 1616 | - | 0 | 15.2 |
| BR 1614 | - | 0 | 19.8 |
| BR 1618 | - | 0 | 14.3 |
| BR 1593 | + | 18 | 15.5 |
| BR 1617 | - | 0 | 12.9 |
| BR 1619 | - | 0 | 13.7 |
| BR 1235 | + | 10.5 | 14.7 |
| BR 1311 | - | 0 | 14.2 |
| BR 1287 | + | 11.7 | 10.6 |
| BR 1309 | - | 0 | 13.5 |
| BR 1499 | - | 0 | 13.7 |
| BR 1507 | - | 0 | 13.5 |
| BR 1303 | - | 35 | 15.8 |
| BR 1319 | - | 0 | 16.3 |
| BR 3010 | + | 10.5 | 15.2 |
| BR 3014 | + | 11.6 | 8.7 |
| BR 3042 | + | 10.1 | 23.2 |
| BR 3045 | + | 10.1 | 22.7 |

### *5.3.2 Optimisation*

Both Bst 2.0 and Bst 3.0 DNA polymerases (New England Biolabs), along with their associated buffers as well as the WarmStart® Colorimetric LAMP 2x Master Mix were tested. In addition, the effects of adding Betaine, different dyes (e.g., SYBR Green), varying incubation periods, and adding different concentrations of guanidine hydrochloride were assessed.

To validate the method, the amplified products were analysed by agarose gel electrophoresis and a PCR for the *stb* gene (Blanco et al., 1997) was done.

# 6 Results

## *6.1* Campylobacter coli *DNA extraction*

Two DNA extraction methods were developed and evaluated.

### *6.1.1 Filtration*

A volume of 15 ml effluent was passed through 11 and 0.22 µm pore size filters. The 0.22 µm filter paper is then transferred into a new 15 ml tube and 450 µl lysis buffer is added. The mixtures is then vigorously shaken with the hands until all content from the filter paper is mixed with the lysis solution. To the solution, 50 µl of 10% SDS was added plus or minus proteinase K. This mixture was incubated at 98°C for 10 minutes and then cooled on ice for 4 minutes, followed by another 5 minutes of incubation at 98°C. After incubation, AMPure Magnetic beads were added to the tube at a 1:1 ratio. The tube was then placed in a magnetic rack and the fluid discarded. The magnetic beads with bound DNA were then washed with 70% ethanol and dried for 2 minutes. The DNA was eluted with 100 µl of $H_2O$. This DNA in water was then used as the template for PCR and LAMP assays.

### *6.1.2 BentoLab*

For this extraction method, 12 ml of effluent was distributed into 6 x 2 ml tubes. After a light spin at 800 rpm for 1 minute, the supernatant from each tube was transferred into a new 2 ml tube. The supernatant was spun for 30 minutes at 13,500 rpm. This second supernatant was discarded, and 75 µl of lysis buffer (0.1 M EDTA, 0.1 M Tris, and 0.2 M NaCl) was added to each tube. Once the pellets were dissolved, the resulting solution was combined into a single tube. Then, 50 µl of 10% SDS was added. The solution was heated for 10 minutes at 98°C, placed on ice for 3 minutes, and then heated again at 98°C for 6 minutes. The tube was spun at 13,500 rpm for 3 minutes, and the supernatant was transferred to a fresh tube. AMPure Magnetic beads were added to the tube at a 1:1 ratio. The tube was then placed in a magnetic rack to discard the fluid, then washed twice with 70% ethanol. After 2 minutes drying time, the DNA was eluted with 100 µl of $H_2O$.

An additional step was to run the treatment groups through the Zymo spin column to further enhance DNA purity.

## *6.2* Campylobacter coli *results*

### *6.2.1 Optimisation results*

Effluent samples collected from the first piggery were not subjected to culture and PCR method. In contrast, samples from the second piggery were analysed using both culture and PCR methods, which served as the gold standard for *C. coli* detection.

Even though the samples from this second piggery (Farm II) were positive for *C. coli* by culture, the original conventional PCR and LAMP assays failed to yield a positive result, regardless of the extraction method. However, replacing the DNA polymerase (Roche) with the Platinum II Taq Hot-Start DNA Polymerase in the conventional PCR assay and replacing the Bst 2.0 with Bst 3.0 DNA polymerase in the LAMP assay resulted in positive amplification in agreement with the culture results (Table 4).

Effluent from a different piggery (Farm III), as well as the one from Farm I, was used for spiking with 1 ml of $10^8$ to $10^6$ CFU of *C. coli* in PBS and subjected to different DNA extraction methods. Results

indicated that both rough and fine filtering methods, followed by DNA cleanup with AMPure Magnetic beads, were successful. The Bst 2.0 polymerase yielded a positive result for only one sample extracted using the Bento Lab method, followed by Zymo column purification (Table 5). Proteinase K added to the filtration method did not produce any positive results.

Figure 6 shows that despite a larger starting effluent volume for DNA extraction, the Roche and Bst 2.0 DNA polymerases do not yield positive amplification results in the conventional and LAMP assays, respectively. The only exception was when the effluent from Farm I was spiked with $10^6$ *C. coli*, and DNA was extracted using the Bento Lab method, followed by filtration via a Zymo column. In addition, DNA extracted with the Bento Lab method, without treatment with the Zymo column, produced positive results in the LAMP assay using Bst 3.0, indicating that this polymerase removes the need for the extra treatment step with the Zymo column.

In conclusion, the Platinum Taq II DNA polymerase enhances the sensitivity of conventional PCR for *C. coli*, while the Bst 3 DNA polymerase enhances the sensitivity of the LAMP assay for detecting this bacterial species. This study has shown that effluent testing with LAMP assay can be used for POM monitoring of pathogens in pig herds. The improved results are presumably due to the fact that Platinum Taq II DNA polymerase and Bst 3.0 DNA polymerase are better able to tolerate the polymerase inhibitors present in effluent, as compared to the polymerase enzymes used in the original reported methodologies of these assays

Table 4. Results of DNA extraction, conventional PCR and LAMP assay for detection of *C. coli* in the effluent collected from Farm II.

| Shed | Cultural & PCR confirmation (Bacterial plate Count) | Starting volume for DNA extraction | DNA extraction method | Conventional PCR results/Polymerase used (on DNA from effluent extraction) | Lamp results (with Bst 2.0 enzyme) | Lamp results (with Bst 3.0 enzyme) |
|---|---|---|---|---|---|---|
| Grower 4 | Yes (TNC*/0.1 mL) | 16 mL | Lab based + Zymo colunm | Positive/Platinum Taq II DNA Polymerase | Negative | Positive |
| Dry sows | Yes (33-71/0.1 mL) | 16 mL | Lab based + Zymo colunm | Positive/Platinum Taq II DNA Polymerase | Negative | Positive |
| Grower 4 | Yes (TNC/0.1 mL) | 16 mL | Lab based method | Positive/Platinum Taq II DNA Polymerase | Negative | Positive |
| Dry sows | Yes (33-71/0.1 mL) | 16 mL | Lab based method | Positive/Platinum Taq II DNA Polymerase | Negative | Positive |
| Grower 4 | Yes (TNC/0.1 mL) | 15 mL | QiaAmp power faecal Pro Kit | Positive/Platinum multiplex Master mix | Negative | Positive |
| Dry sows | Yes (33-71/0.1 mL) | 15 mL | QiaAmp power faecal Pro Kit | negative/Platinum multiplex Master mix | Negative | Positive |
| Weaning | Yes (56-58/0.1 mL) | 50 mL | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive |
| Grower 1 &2 | Yes (82-105/0.1 mL) | 50 mL | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive |
| Grower 4 | Yes (TNC/0.1 mL) | 50 mL | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive |
| Dry sows | Yes (33-71/0.1 mL) | 50 mL | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive |
| Grower 5 | Yes (TNC/0.1 mL) | 50 mL | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive |

* TNC = too numerous to count

Table 5. Detection of *C. coli* from effluent collected from piggery 3 and spiked in.

| Sample ID | Shed | Starting volume for DNA extraction | DNA extraction method | Conventional PCR results/Polymerase used (on DNA from effluent extraction) | LAMP results (with Bst 2.0 enzyme) | LAMP results (with Bst 3.0 enzyme) | Gel electrophoresis (for verification of Bst 3.0 product) |
|---|---|---|---|---|---|---|---|
| Treated E108 | Dry sows | 12 mL | Bento Lab + Zymo colunm | NA | Negative | Positive | Positive |
| Treated E107 | Dry sows | 12 mL | Bento Lab + Zymo colunm | NA | Negative | Positive | Positive |
| Treated E106 | Dry sows | 12 mL | Bento Lab + Zymo colunm | NA | Positive | Positive | Positive |
| NS E108 | Dry sows | 12 mL | Nucleo spin soil DNA kit | NA | Negative | Positive | Positive |
| NS E107 | Dry sows | 12 mL | Nucleo spin soil DNA kit | NA | Negative | Positive | Positive |
| NS E106 | Dry sows | 12 mL | Nucleo spin soil DNA kit | NA | Negative | Positive | Positive |
| RFA bead clean | Pooled | 15 mL | Rough Filter | Negative/Roche Taq DNA Polymerase | Negative | Positive | Positive |
| RFB bead clean | Pooled | 15 mL | Rough Filter + Proteinase K | Negative/Roche Taq DNA Polymerase | Negative | Negative | Negative |
| FFA bead clean | Pooled | 15 mL | Fine Filter | Negative/Roche Taq DNA Polymerase | Negative | Positive | Positive |
| FFB bead clean | Pooled | 15 mL | Rough Filter + Proteinase K | Negative/Roche Taq DNA Polymerase | Negative | Negative | Negative |

Treated stands for effluent samples spiked ($10^8$ to $10^6$). NS stands for not spiked. RFA stands for the rough filtration method without polymerase. FFA: fine filtration without proteinase K. RFB: rough filtration with proteinase K. FFB: fine filtration with proteinase K. NA: not done. Pooled: effluent from Porker, Dry sows and Farrows were pooled.

| Sample ID | Piggery | Shed | Cultural confirmation (Bacterial plate count) | Starting volume for DNA extraxtion | DNA extraction method | Conventional PCR results. Polymerase used | LAMP reults (with Bst 2.0 enzyme) | LAMP reults (with Bst 3.0 enzyme) | LAMP gel results (for verification of |
|---|---|---|---|---|---|---|---|---|---|
| NS weaning | 2 | Weaner | Yes (56-58/0.1 ml) | 50 ml | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive | Positive |
| NS G1/G2 | 2 | Grower 1 + 2 | Yes (82-105/0.1 ml) | 50 ml | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive | Positive |
| NS G4 | 2 | Grower 4 | Yes (TNC/0.1 ml) | 50 ml | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive | Positive |
| NS D5 | 2 | Dry sows | Yes (33-71/0.1 ml) | 50 ml | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive | Positive |
| NS G5 | 2 | Grower 5 | Yes (TNC/0.1 ml) | 50 ml | Nucleo spin soil DNA kit | negative/Roche Taq DNA Polymerase | Negative | Positive | Positive |
| Treated G4 | 2 | Grower 4 | Yes (TNC*/0.1 ml) | 16 ml | Bentolab method + Zymo column treatment | Positive/Platinum Taq II DNA Polymerase | Negative | Positive | Positive |
| Treated D5 | 2 | Dry sows | Yes (33-71/0.1 ml) | 16 ml | Bentolab method + Zymo column treatment | Positive/Platinum Taq II DNA Polymerase | Negative | Positive | Positive |
| G4 untreated | 2 | Grower 4 | Yes (TNC/0.1 ml) | 16 ml | Bentolab method | Positive/Platinum Taq II DNA Polymerase | Negative | Positive | Positive |
| D5 untreated | 2 | Dry sows | Yes (33-71/0.1 ml) | 16 ml | Bentolab method | Positive/Platinum Taq II DNA Polymerase | Negative | Positive | Positive |
| G4 QIaAmo | 2 | Grower 4 | Yes (TNC/0.1 ml) | 15 ml | QIaAmp power faecal Pro Kit | Positive/Platinum multiplex Master mix | Negative | Positive | Positive |
| D5 QIaAmo | 2 | Dry sows | Yes (33-71/0.1 ml) | 15 ml | QIaAmp power faecal Pro Kit | negative/Platinum multiplex Master mix | Negative | Positive | Positive |
| Treated E10^8 | 1 | Dry sows spiked E10^8 | Not done | 15 ml | Bentolab method* + Zymo column treatment | Not tested with conventional PCR | Negative | Positive | Positive |
| Treated E10^7 | 1 | Dry sows spiked E10^7 | Not done | 15 ml | Bentolab method + Zymo column treatment | Not tested with conventional PCR | Negative | Positive | Positive |
| Treated E10^6 | 1 | Dry sows spiked E10^6 | Not done | 15 ml | Bentolab method + Zymo column treatment | Not tested with conventional PCR | Positive | Positive | Positive |

Figure 6. Nucleo spin soil DNA kit, QiaAmp Power Faecal Kit and Bento Lab with and without Zymo column treatment were compared as well as the Roche and Platinum Taq polymerase in the conventional PCR.

### 6.3 Can *Actinobacillus pleuropneumoniae* be detected in Effluent?

An *A. pleuropneumoniae* was revived for the spiking part of the experiment. A tenfold serial dilution was prepared (from $10^8$ to $10^{-1}$) from the overnight culture in PBS with McFarland standard 2 as the starting point. The bacterial concentrations were confirmed by plating 0.1 ml of the $10^4$ to $10^2$ dilutions onto BASN agar plates and counting the colonies after overnight incubation at 37°C. Fourteen ml of effluent was spiked with 1 ml of this serially diluted *A. pleuropneumoniae* and DNA was extracted using the BentoLab DNA extraction method with and without the Zymo column, and with the Nucleon spin extraction kit. The extracted DNA was tested with an *A. pleuropneumoniae-specific* PCR (Turni et al, 2014). Amplification products were visualised via agarose gel electrophoresis and results compared. Results indicated that treatment of the extracted DNA following the BentoLab method had a positive effect on the detection of the *A. pleuropneumoniae* (Figure 7). The Bento Lab extraction method only yielded positive results when the samples were diluted at least 1:1 for PCR. The spiked samples treated with the Zymo column showed strong bands at $10^6$ to $10^8$ cfu/ml.

The sensitivity of the PCR for the *A. pleuropneumoniae* spiked effluent was $10^6$ cfu/ml.

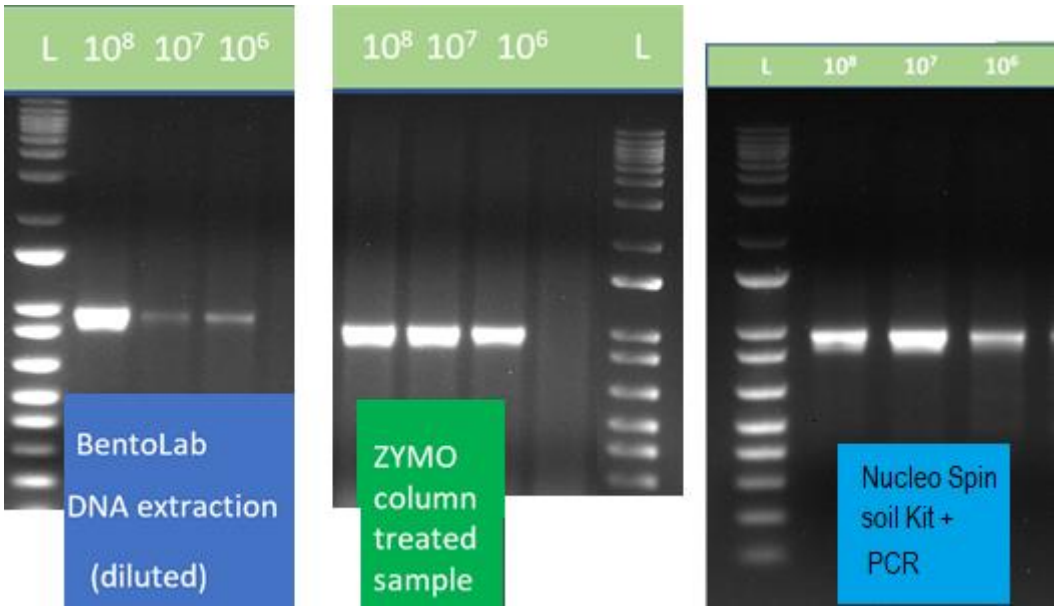



Figure 7. Results of effluent spiked with a 10-fold dilutions of *Actinobacillus pleuropneumoniae* ($10^8$ to $10^{-1}$) and extracted with the BentoLab DNA extraction with and without the Zymo column, and with the Nucleon spin extraction kit and the extracted DNA was tested with an *A. pleuropneumoniae-specific* PCR.

As effluent is a complex matrix with high levels of PCR inhibitors, the *A. pleuropneumoniae* multiplex serotyping PCR was modified to the Platinum II Taq Hot-Start DNA Polymerase. When tested with serial dilutions of *A. pleuropneumoniae* in PBS, this polymerase achieved a detection limit of $10^2$ CFU/mL (Figure 8).

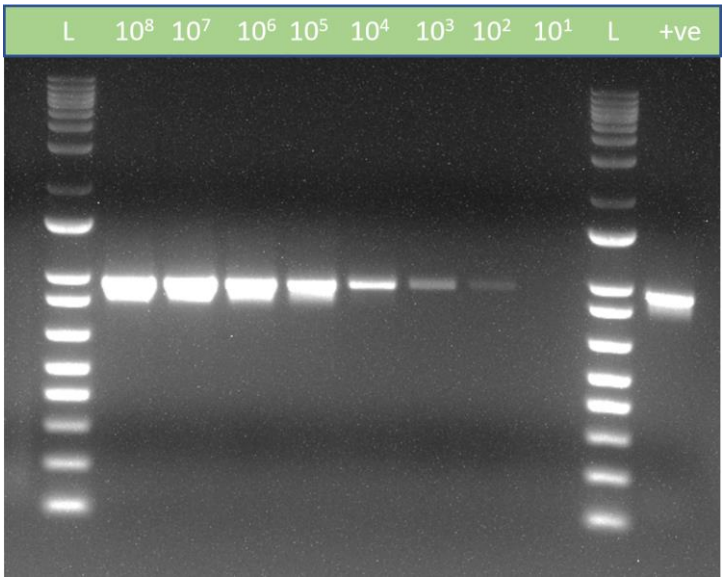



Figure 8. Dilution of *A. pleuropneumoniae* tested with the altered PCR.

### *6.3.1 The improved species specific PCR*

The updated species specific PCR for *A. pleuropneumoniae* is listed in Table 6 with primer pairs LPF (5'-AAG GTT GAT ATG TCC GCA CC-3') and LPR (5'-CAC CGA TTA CGC CTT GCC A-3') amplifying a 951 bp region from the outer membrane lipoprotein as described by Gram and Ahrens (1998).

Table 6. Modified species-specific PCR assay for detection of *A. pleuropneumoniae*

| Ingredient | Vol. µl | Final concentration | Cat. No. |
|---|---|---|---|
| 5 x Platinum II PCR Buffer | 5 | 1 µl | 14966005 |
| dNTPs (1.25 mM each) | 4 | 0.2 mM each | |
| Forward primer (10µM) | 0.5 | 0.2 µM | |
| Reverse primer (10µM) | 0.5 | 0.2 µM | |
| $H_2O$ | 14.8 | | |
| Platinum II Taq HS DNA Polymerase | 0.2 | | |
| Total | **24** | | |

The PCR was run on an Eppendorf thermal cycler with the following cycling conditions: an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 66°C for 20 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. Amplification products were visualised by electrophoresis using 10 $\mu$l of the amplified product on 1% agarose gel (Progen Pharmaceutical, Darra, Australia) containing 50 ng $ml^{-1}$ ethidium bromide in TAE buffer (0·04 mol $l^{-1}$ Tris-acetate, 0·01 mol $l^{-1}$ EDTA) at 70 V for 2 h and photographed under UV illumination.

## *6.4* Results of the LAMP assay for *A. pleuropneumoniae*

### *6.4.1* *Optimisation results*

The LAMP assay published by Yang et al. (2009) is based on detection of the apxIVA gene and uses Bst DNA polymerase large fragment for amplification and the SYBR Green I for immediate detection. The aim of the project was to first establish the same test. After initial attempts in optimisation using a different polymerase, $MgSO_4$ concentrations, Betaine concentration and the amplification time, it was decided to test the New England BioLab WarmStart Colorimetric LAMP Master Mix (New England Biolabs, Massachusetts, USA) as it is more suitable for an on-farm assay,

Different amplification times were tested. The LAMP assay was first evaluated using *A. pleuropneumoniae*-spiked PBS, where it achieved a detection limit of $10^3$ CFU/mL (Figure 9 and Figure 10).

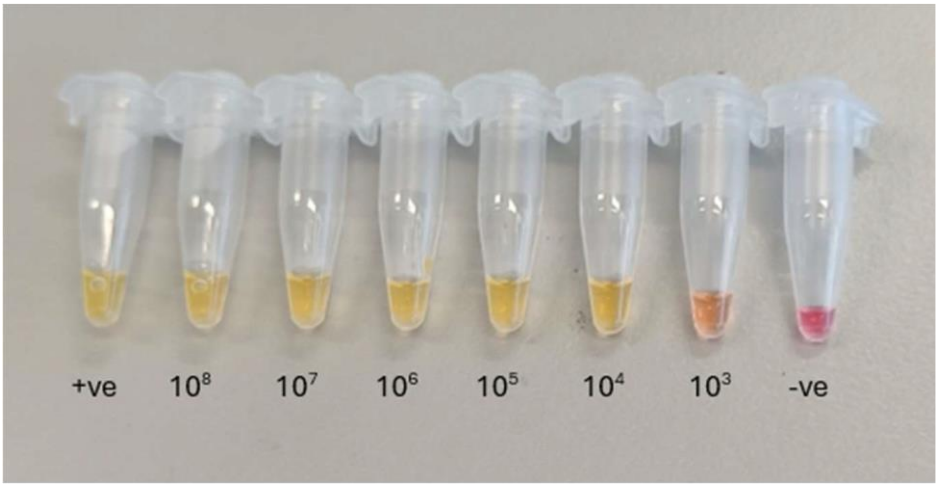



Figure 9. LAMP assay run for 45 minutes for a freshly extracted spike *A. pleuropneuomiae* DNA dilution series in PBS.

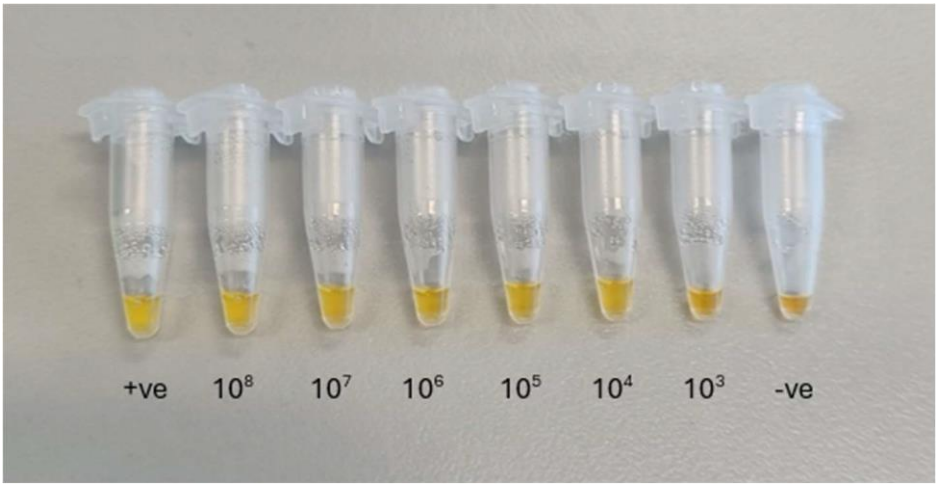



Figure 10. 60 minutes of LAMP assay on spike *A. pleuropneuomiae* DNA dilution series in PBS. Illustrates that all the samples turned positive.

### *6.4.2 Sensitivity results*

The sensitivity test was then performed with spiked-in effluent with different reaction time tested. Results for the spiked-in effluent were that the optimal reaction time was 30 minutes, which gave a limit of detection of 2.88 x $10^3$ cfu/ml (Figure 11). The electrophoresis of the amplified LAMP product confirmed that the colour change was real amplification (Figure 12).

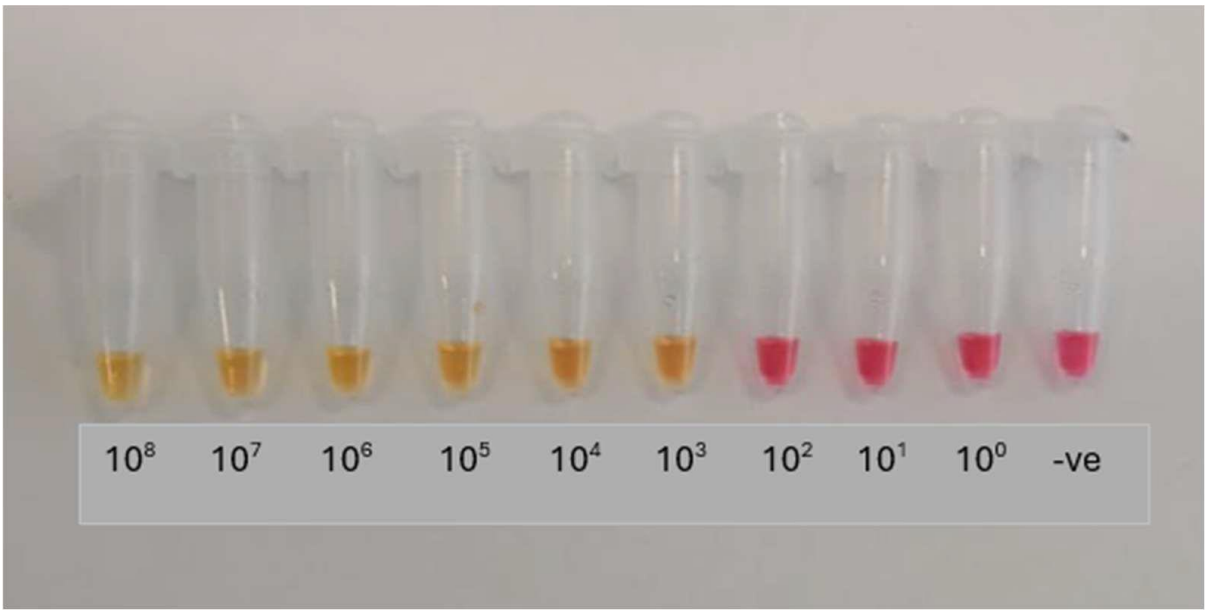



Figure 11. Effluent samples spiked-in with different dilutions of *A. pleuropneumoniae* to determine the sensitivity of the *A. pleuropneumoniae* LAMP assay.

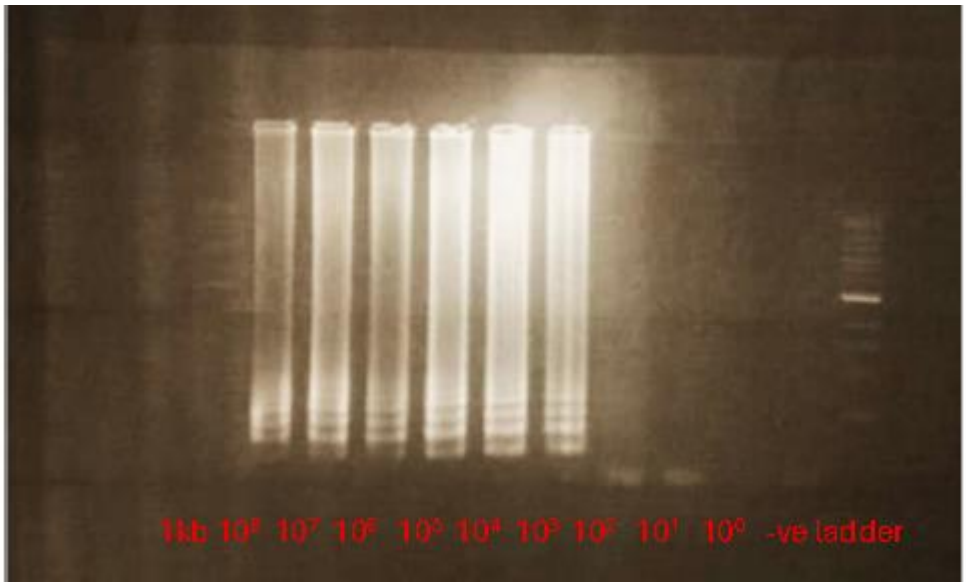



Figure 12. The lanes depicting DNA concentrations $10^8$ to $10^3$ show bands, while the remaining concentrations show no amplification.

### *6.4.3 Specificity results*

For the specificity, we spiked the effluent with related species (Table 2-1). Two different extractions were used to see if the related species gave any false-positive results: first, by suspending the bacterial colonies in water and performing a crude DNA extraction via the boiling and cooling method without the use of effluent. Second, by spiking effluent with related bacterial species and extracting the DNA via the magnetic bead extraction method described in section 4.1.4 of this report. The spiking was done as described above with 1 ml of $10^7$ cfu/ml of each bacterial species mixed with 14 ml of effluent.

When using the heating and boiling method, all related species samples tested negative, and only the positive controls yielded positive results. The negative is displayed as a reddish colour, while a positive is yellow (Figure 13 and Figure 14).

The extracted DNA via the magnetic bead extraction from the spiked effluent produced negative results as well (Figure 14 and Figure 15).

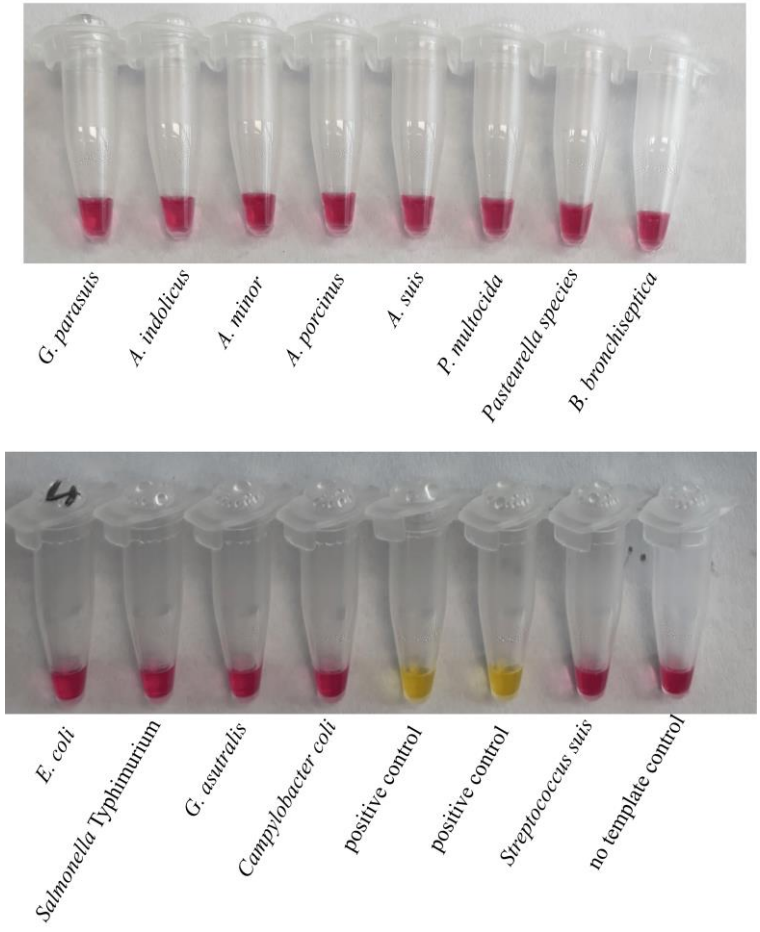



Figure 13 Crude DNA extraction from related species tested in the LAMP assay

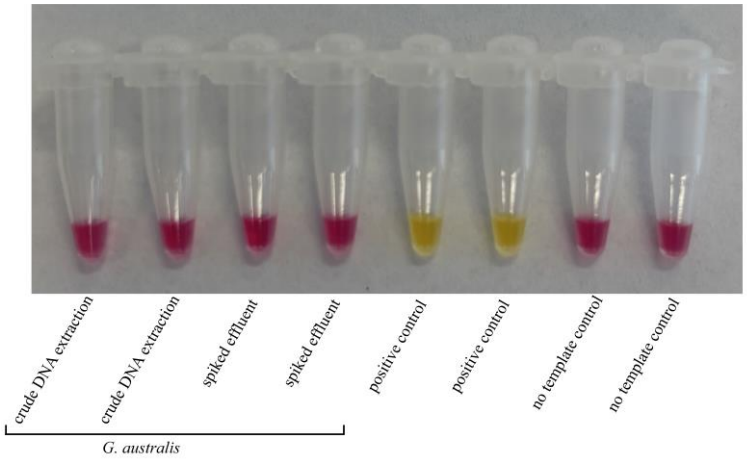



Figure 14 Spiked effluent with *G. australis* (magnetic bead extraction) and crude DNA extraction (boiling and ice from colony mixed with water).


Figure 15 Spiked effluent with related species and DNA extraction via magnetic bead extraction.

The samples were run on a 1% agrose gel to make sure there are no positive amplifications (Figure 16).

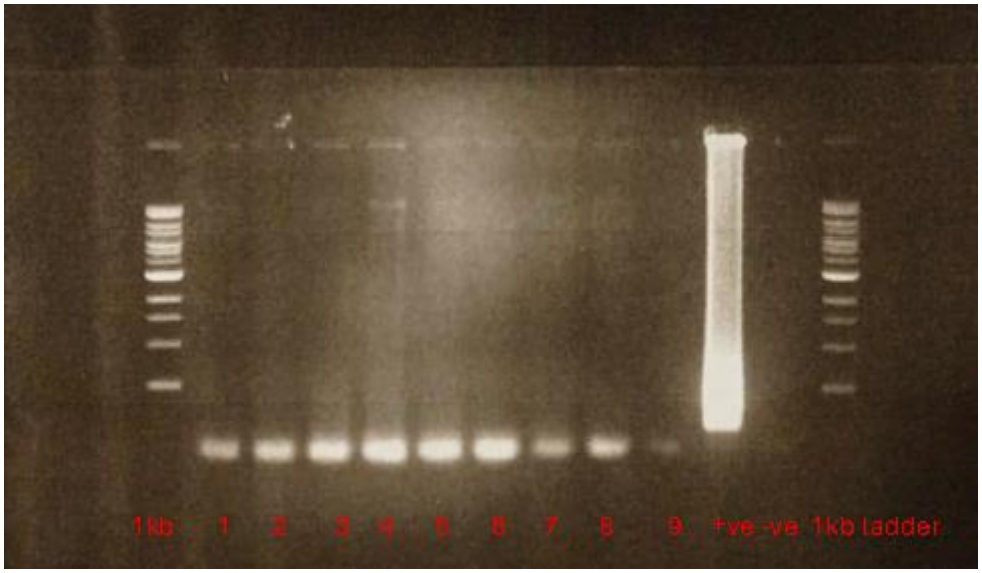



Figure 16. 1% Agarose gel reflecting the specificity results. The gel is labelled with a 1kb DNA ladder, positive and negative controls, and some of the related species from the specificity test, labelled from 1 to 9 lanes. 1 denotes *H. parasuis,* 2 *A. indolicus*, 3 *A. minor*, 4 *A. porcinus*, 5 A. suis, 7 *P. multocida*, 8 *Pasteurella* species, 9 *G. parasuis*.

### *6.4.4 Final assay*

The reaction was prepared to a final volume of 25 µl, consisting of 12.5 µl of WarmStart Colorimetric LAMP 2 x Master Mix (New England Biolabs, Massachusetts, USA), a total of 0.2 µM of F3 and B3 primers, 1.6 µM of FIP and BIP primers and 0.4 µM of LF and LB primer (Table 7), and 1 µl of DNA. $H_2O$ was used to make up the 25 µl. The mix was then incubated at 63°C for 30 min in a PCR machine made for use on farm (Bento lab) and then heated at 80°C for an additional 10 min to stop the reaction.

Table 7. Primers used for the final assay:

| Primer name | Primer sequence |
|---|---|
| F3 | TTGATGCCGGTGCGGGTA |
| B3 | CCAAAGTTCGGATAAAT |
| FIP | CGTAGCCACCTCTTAGAATATCATTTTTTGGTTAATGGCGGTAATGG |
| BIP | ATCTTTAGCAAAGGACACGGACAGTTTTAGGTGTCGATATCTCTTGC |
| LF | TTTGCCGCCGATGAGGGT |
| LB | CGTTTATGAAGATACCAA |

## *6.5* Escherichia coli *results*

### *6.5.1 Optimisation results*

The effluent from three piggeries was plated on culture plates. The isolated *E. coli* strains via culture were tested with previously published PCRs for different toxin genes, including the *stb* gene (Blanco et al., 1997). Piggery 1 had 4 positive isolates for the *stb* gene. Piggery 2 did not have any positive *E. coli* isolates from the sampled central pond. However, when the effluent from piggery 2 was tested

directly, the *stb* gene was detected by PCR. From piggery 3 two samples were positive for other virulence factors that are normally associated with *E. coli* (Figure 8). Because the effluent samples were STb-positive by PCR, they were hard-spun to remove background *E. coli* for the sensitivity and spiking-with-related-species experiments.


Figure 17. *E. coli* isolate from effluent.

Table 8. Results of culture and PCR test for *stb* gene on effluent samples

| Source | No. of *E. coli* isolates | Piggery | No. of STb positive *E. coli* |
|---|---|---|---|
| Cultured isolates from effluent | 4 | 1 | 4 |
| | 7 | 2 | 0 |
| | Not done | 3 | NA |
| Source | No. of effluent samples | Piggery | No. of STb positive effluent samples |
| Direct PCR on effluent | 1 | 1 | 0 |
| | 1 | 2 | 1 |
| | 2 | 3 | 0 |

As mentioned in the materials and methods, different primer sets were designed for the detection of the *stb* gene on farm and tested systemically. Table 9 and Table 10 describe a snapshot of some the tests done from which LAMP primer set 20 was chosen as the ideal primer set.

The sets that looked promising were set 6, 20 and 32. These were tested against a range of *E. coli* isolates with and without the *stb* gene according to qPCR for *A. pleuropneumoniae* (Table 6-5). Set 6 gave the least concordance with the qPCR (Table 6-6).

Table 9 Comparison of LAMP detection results and qRT-PCR confirmation for *E. coli* (*STb* gene)

| BR numbers | qPCR Cassey primers (Ct) | Qubit measurement | LAMP set 6 | LAMP set 20 | LAMP set 32 |
|---|---|---|---|---|---|
| 1602 | 10.5 | 21.7 | + | + | + |
| 1606 | 18 | 12.4 | - | + | + |
| 1601 | 0 | 20 | - | - | - |
| 1616 | 0 | 20.9 | - | - | - |
| 1614 | 0 | 19.8 | - | - | - |
| 1618 | 0 | 14.3 | - | - | - |
| 1593 | 18 | 15.5 | - | + | + |
| 1617 | 0 | 16.8 | - | - | - |
| 1619 | 0 | 13.7 | - | - | - |
| 1235 | 10.5 | 14.7 | + | + | + |
| 1311 | 0 | 14.2 | - | - | - |
| 1287 | 11.7 | 13.6 | + | + | + |
| 1309 | 0 | 13.5 | - | - | - |
| 1499 | 0 | 13.7 | - | - | - |
| 1507 | 0 | 13.5 | - | - | - |
| 1303 | 35 | 15.8 | + | + | + |
| 1319 | 0 | 16.3 | - | - | - |
| 3010 | 10.5 | 18.5 | + | + | + |
| 3014 | 11.6 | 8.7 | + | + | + |
| 3042 | 10.1 | 23.2 | + | + | + |
| 3045 | 10.1 | 22.7 | + | + | + |

Table 10 Performance summary of designed primer sets in detecting *E. coli* (*stb* gene).

| Primer Set | Positive strains detected by LAMP (n/total) | Concordance with q-PCR (%) |
|---|---|---|
| Set 6 | 8/10 | 80% |
| Set 20 | 10/10 | 100% |
| Set 32 | 10/10 | 100% |

The ideal reaction time for these primer sets was then optimised by testing the assay on serially diluted STb positive *E. coli* isolate (BR1602) and extracting the DNA via crude DNA extraction method from PBS suspended bacteria spiked in effluent (Figure 18. Panels A to D). Figure 18 A and B depict set 20 amplifying for 30 and 35 minutes with a strong colour change for the dilutions from $10^8$ to $10^4$ cfu/ml at 35 minutes. Set 6 and 32 only reached sensitivities of $10^7$ and $10^6$ cfu/ml, respectively and even these colour changes were very weak.

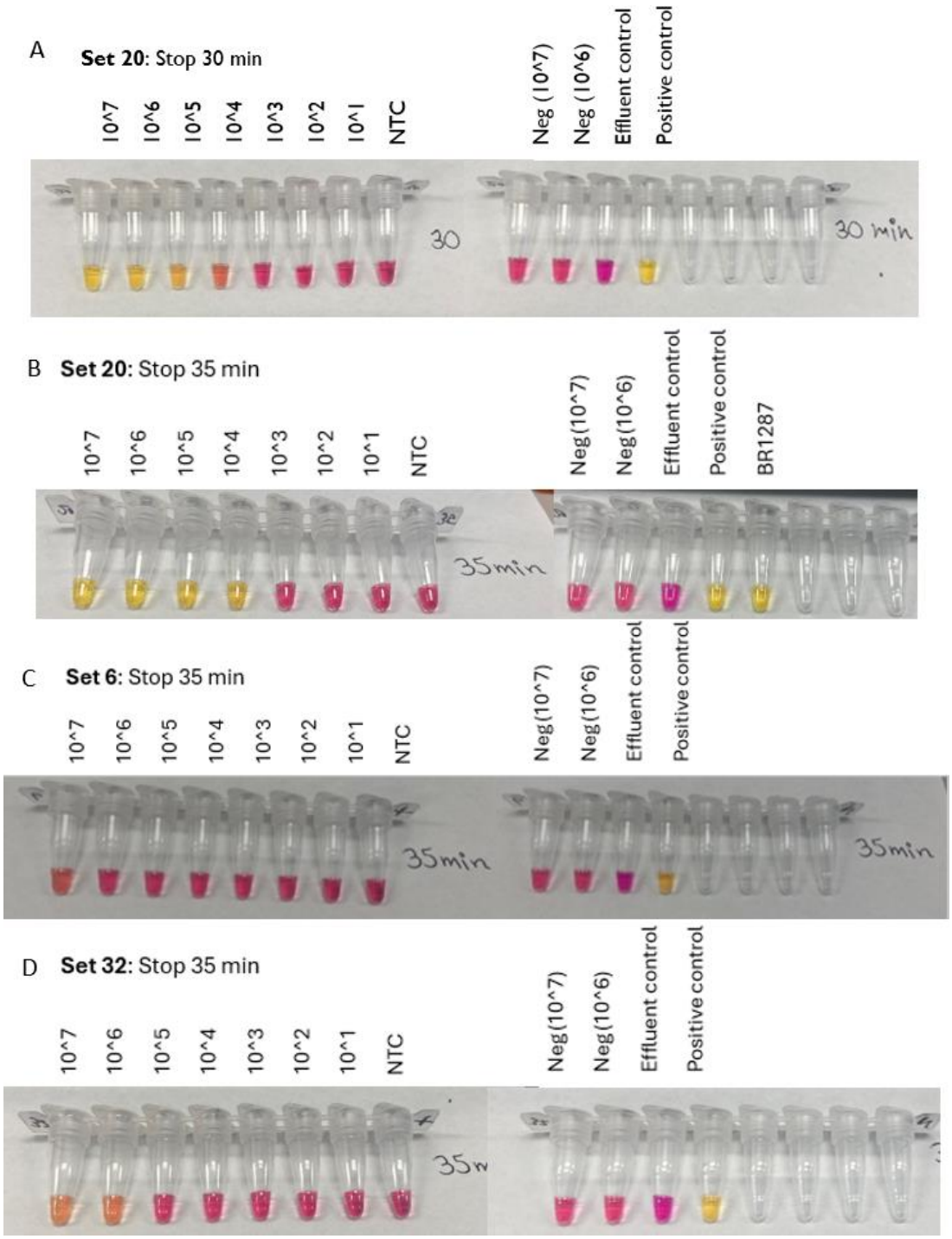



Figure 18. Comparing amplification time of the LAMP for different primer sets. A) running time of 30 minutes for primer set 20, B) running time of 35 minutes for primer set 20, C) running time of 35 minutes for primer set 6 and D) running time of 35 minutes for primer set 32.

### *6.5.2 Sensitivity results for set 20*

All these tests were repeated once set 20 was confirmed as the best primer set for STb detection (Figure 19 and Figure 20). The optimal amplification period was 35 minutes where all colour changes for STb positive dilutions were very strong. Beyond 35 minutes some inconsistent results are

observed with some negative controls changing colour. The test was repeated to test for consistency of results. However, 35 minutes came up again as the ideal time period and the minimum detection rate for set 20 was a sensitivity of 3.52 x $10^4$ cfu/ml (Figure 20).

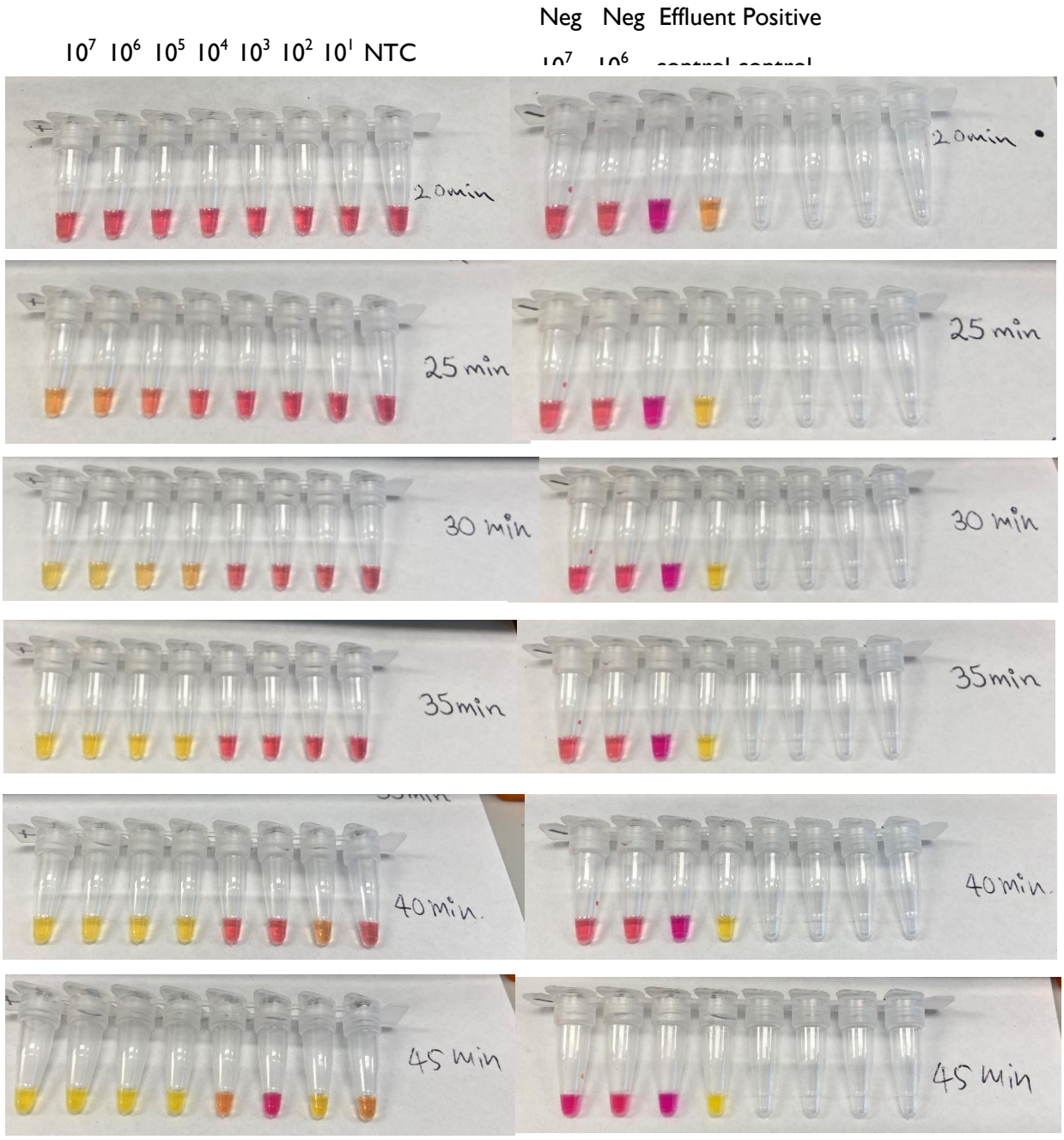


Figure 19. Repeat of amplification times for primer set 20

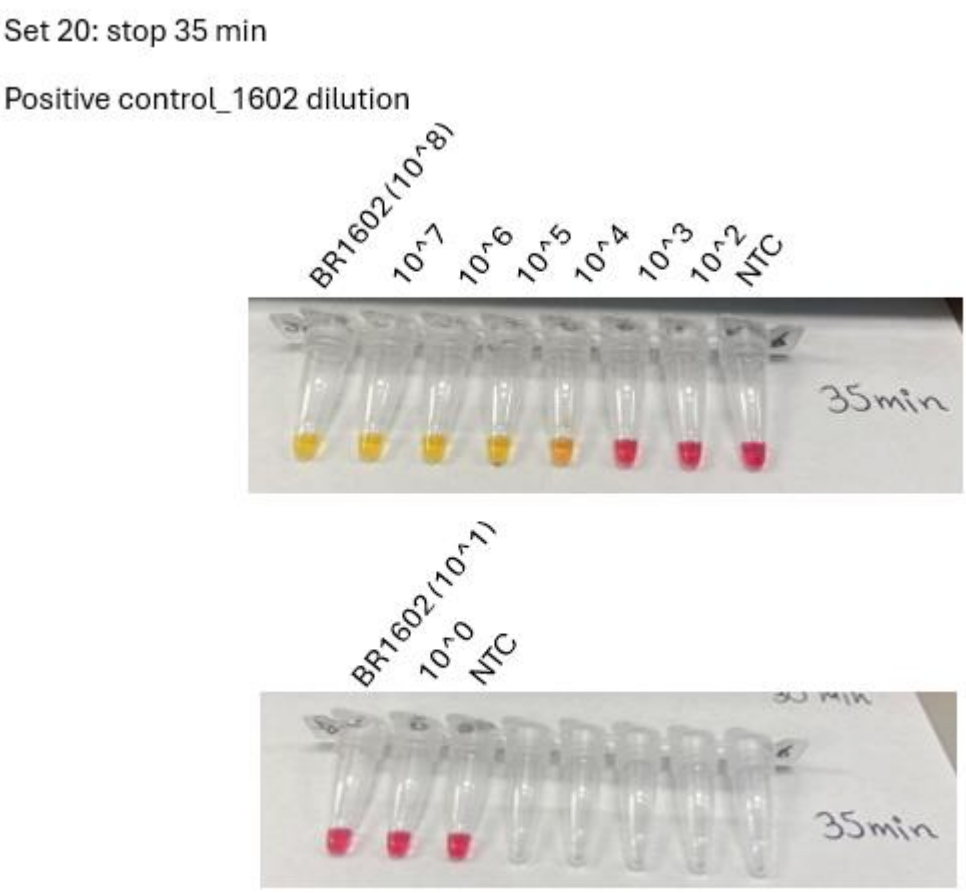
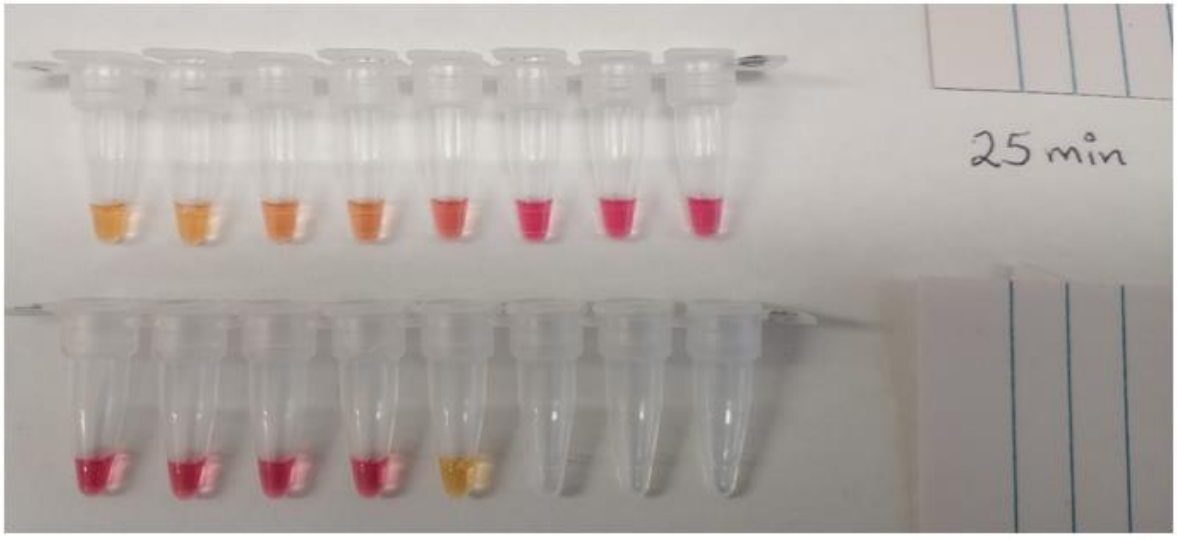



Figure 20. Using the best amplification time to test repeatability.

The result of $10^4$ cfu/ml was not the desired outcome and indicated a need for improvement. After further investigation, a solution was found. The paper by Knox and Geddoe (2024) described a method of using guanidine chloride to improve assay kinetics. They tested guanidine chloride concentrations ranging from 20 to 60 mM to accelerate amplification and enable amplification of lower DNA amounts. Our results from testing different concentrations of guanidine chloride indicated that 40 mM was the optimal concentration. The results are seen in Figure 21. The addition of guanidine chloride not only increased the sensitivity but also decreased the running time. The sensitive with the addition of guanidine chloride was at $2.65 \times 10^2$ cfu/ml, which would be 0.265 cfu/µl.

$10^6$ $10^5$ $10^4$ $10^3$ $10^2$ $10^1$ $10^{-1}$ $10^0$



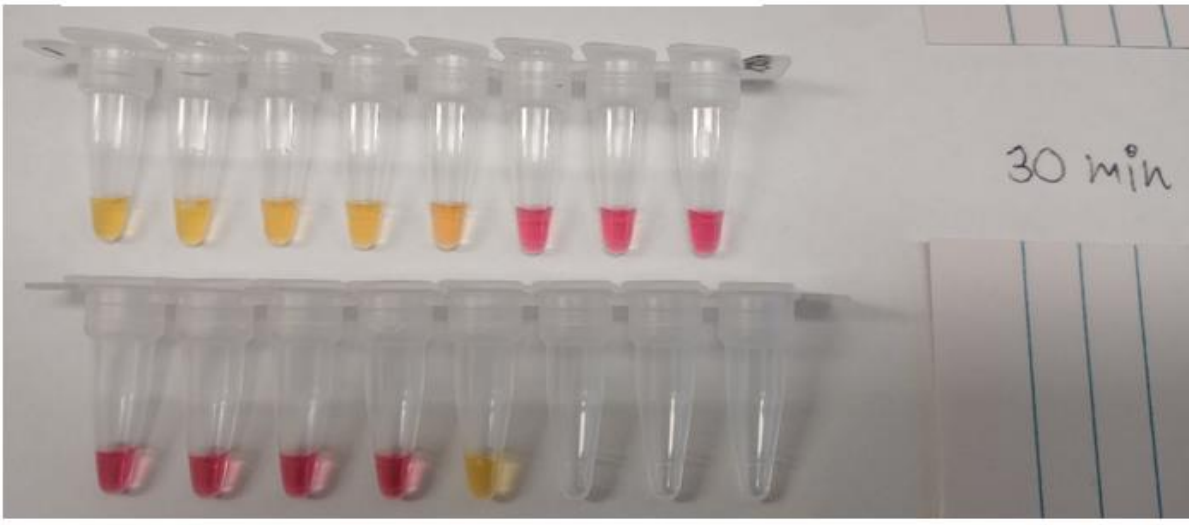

$10^7$ $10^6$ neg effluent $10^7$

Neg Neg effluent $H_2O$ pos

Effluent PBS

**Addition of guanidine chloride**

$10^6$ $10^5$ $10^4$ $10^3$ $10^2$ $10^1$ $10^{-1}$ $10^0$




$10^7$ $10^6$ neg effluent $10^7$

Neg Neg effluent $H_2O$ pos

Effluent PBS

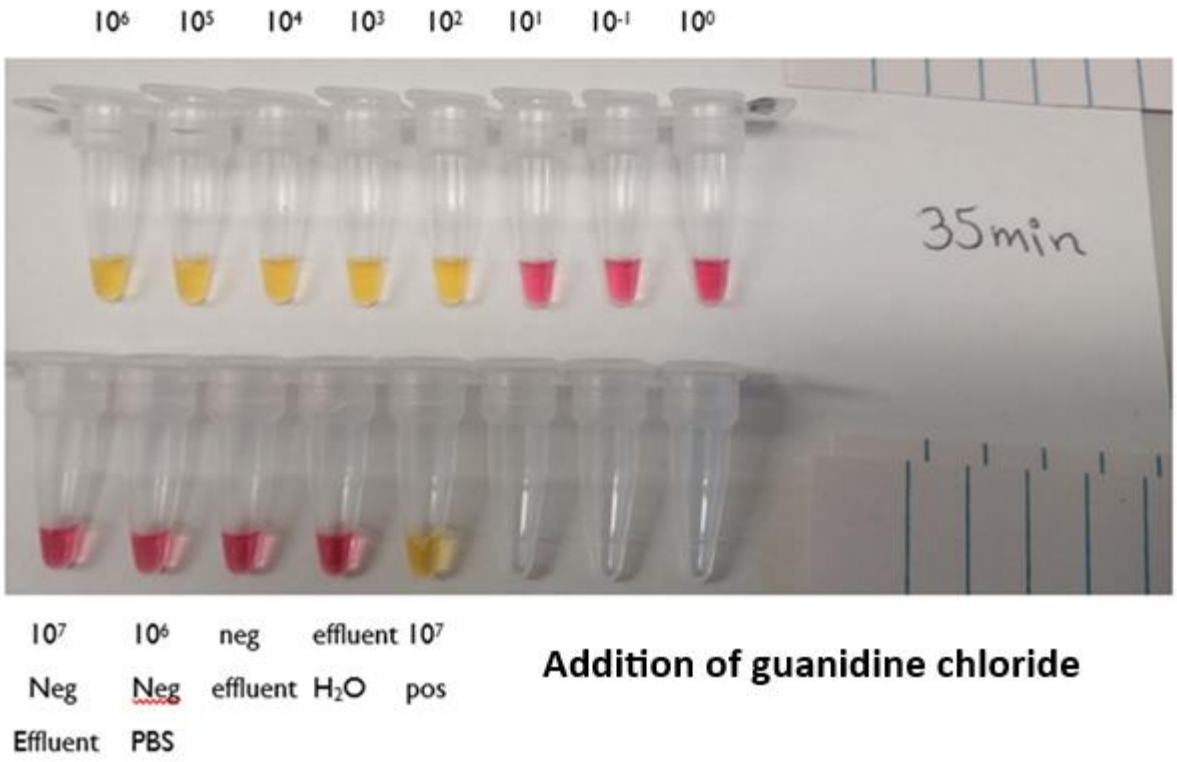



Figure 21. Showing the results when adding 40 mM Guanidine Chloride to the LAMP reaction mix. In this dilution series the sensitivity increases to $10^2$ cfu/ml at running time of 25 to 35 minutes.

### *6.5.3 Specificity results*

The specificity of the assay was tested, and the results demonstrated that the newly developed LAMP assay did not amplify any of the related species tested for when the assay was used with or without the addition of guanidine chloride (Figure 22 and Figure 23).

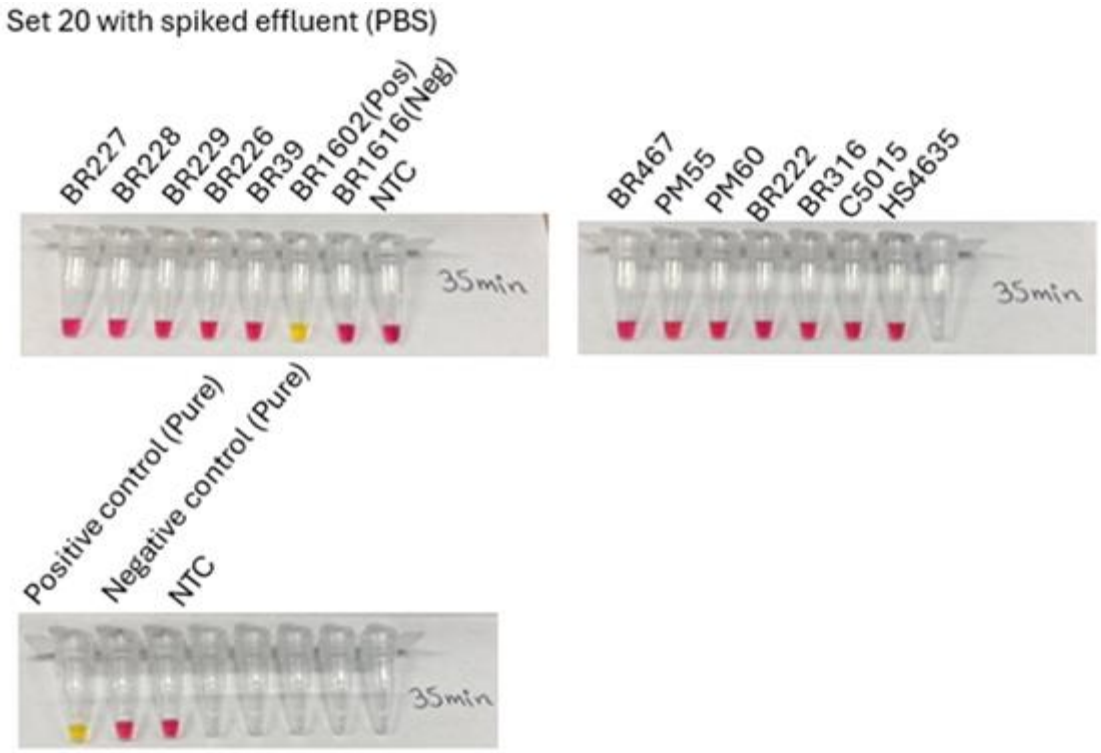
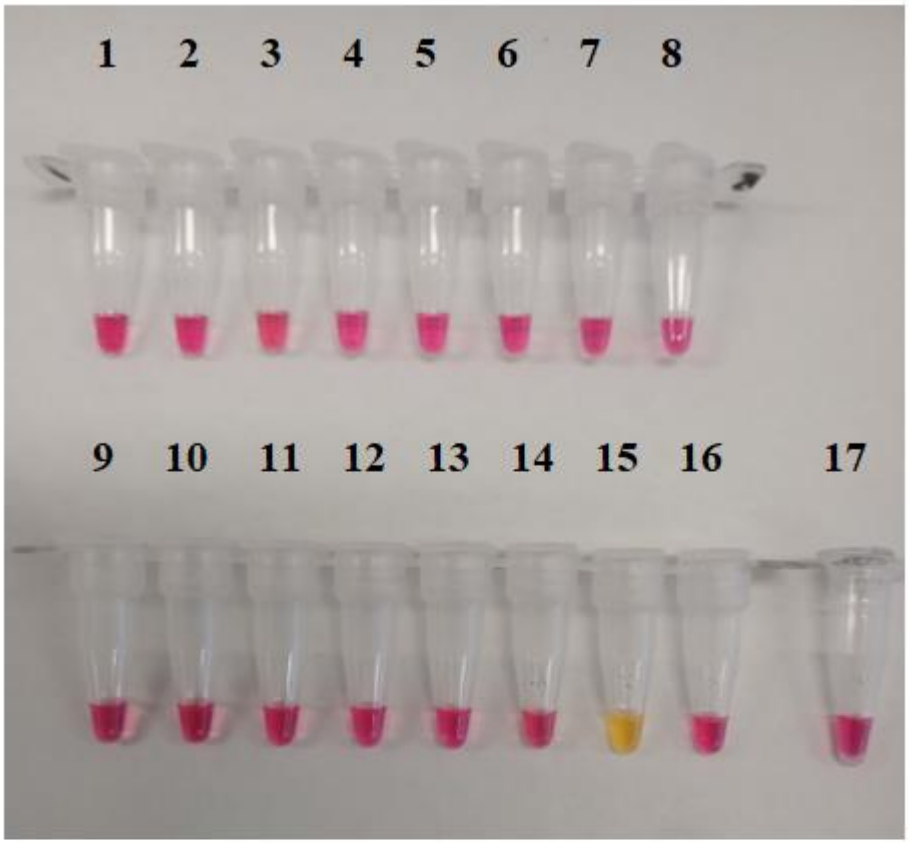



Figure 22 Specificity tested without guanidine chloride.




| Code | | Bacterial species |
|---|---|---|
| 1 | AICC25922 | *E. coli* |
| 2 | CCUG38923 | *Actinobacillus minor* |
| 3 | NCTC10322 | *Pasteurella multocida* |
| 4 | HS 4635 | *Glaesserella australis* |
| 5 | CCUG39029 | *Acintobacillus indolicus* |
| 6 | CCUG221 | *Erysipelothrex rhyussiopathiae* |
| 7 | BR 467 | *Bordetella bronchispetica* |
| 8 | CCUG7984 | *Streptococcus suis* |
| 9 | SSIP683 | *Pasteurella species B* |
| 10 | CCUG38924 | *Actinobacillus porcinus* |
| 11 | CCUG 3712 | *Glaesserella parasuis* |
| 12 | CCUG11624 | *Actinobacillus suis* |
| 13 | BR 258 | *Salmonella enterica* serovar *Typhimurium* |
| 14 | BR 1602 | *E. coli* without STb – negative control |
| 15 | BR 1616 | *E. coli* with STb – positive control |
| 16 | | Effluent control |
| 17 | | Without template (1 µl water) |

Figure 23. Specificity tested with guanidine chloride. The bacteria code for the numbers is under the picture.

### *6.5.4 Final assay*

The assay was set up in a 25 µl volume with 12.5 µl of WarmStart Colourimetric LAMP 2 x Master Mix (New England Biolabs, Massachusetts, USA), a total of 0.2 µM of F3 and B3 primers, 1.6 µM of FIP and BIP primers (Table 11), 40 mM Guanidine Hydrochloride and 1 µl of DNA. The mix is incubated at 63°C for 25 min in a PCR machine designed for use on farms (Bento lab), then heated at 80°C for an additional 10 min to stop the reaction.

Table 11. Primers used were set 20:

| | |
|---|---|
| F3 | GCAATAAGGTTGAGGTGATT |
| B3 | GCAACCATTATTTGGGCG |
| FIP | TTGATTGTGTAGATGCATAGGCATT-ATTTCTTCTTGCATCTATGTTCG |
| BIP | TAGACAAATAGCCAAGGAAAGTTGT-TGCTCCAGCAGTACCATC |

# 7 Discussion

There has been a push to simplify PCR methods so they can be used on the farm. The advantage of on-farm monitoring is that it can be done in real time. Without on-farm sampling, samples need to be sent to a lab, which means that the answer is not available straight away and may be delayed for days.

To simplify DNA amplification assays, isothermal methods have been developed. The important aspect was to ensure that these methods had the same, if not better, sensitivity without sacrificing specificity. A comparison by Craw and Balachandran (2012) found that, among isothermal methods, the LAMP assay performed well, detecting about 5 copies of dsDNA within 60 minutes (depending on the target and assay). It is very specific due to the 4 to 6 primers used. The LAMP assay is the most widely researched assay and widely employed for pathogen detection. For bacteria, the assay has been shown to perform as well as, if not better than, equivalent PCRs. The sensitivity is equivalent to real-time PCRs. The LAMP assay is also highly tolerant of substances that would inhibit PCR. These attributes made it a suitable option for testing effluent, as effluent is rich in inhibitors. The fact that the assay is colourimetric enables detection of DNA amplification through visual changes in the solution's colour. The assays we developed certainly demonstrated this sensitivity and specificity, even exceeding it.

DNA amplification is interfered with by inhibitors in biological and environmental samples. Effluent sampling and DNA extraction are notoriously difficult for downstream DNA amplification (e.g., PCR or LAMP) because the wastewater matrix contains many factors that degrade DNA or inhibit enzymatic reactions. This would explain why the bead assay, which allows washing the DNA to remove inhibitors, worked really well and enhanced the sensitivity. The Zymo column is designed to purify the extracted DNA and has been observed to improve amplification.
The other problem with wastewater is the very small amount of target DNA, due to microbial cells being diluted in large volumes of water (Bilby and Peccia, 2013). This posed the problem that larger volumes needed to be tested and that the assay needed to be very sensitive. For the *Campylobacter* samples, this was achieved either by spinning (BentoLab) or by filtration beforehand and extracting the bacteria from the filter.
Many bacteria in wastewater are Gram-positive, such as *Streptococcus suis*, which have robust cell walls, and standard extractions may fail to lyse them completely, leading to low DNA yield (Albertsen et al., 2015). Hence, it was important to optimise the lysing buffer for Gram-positive bacteria so the platform could be used for other bacteria. We used different lysing buffers, and the one we ultimately used yielded a higher total DNA amount, including background DNA from other bacteria in the effluent (results are not shown).

The Bst polymerases used in LAMP assays exhibit different sensitivities, with the newer Bst 3 polymerase being superior to the Bst 2 polymerase. It was also observed that for the PCR targeting *Actinobacillus pleuropneumoniae*, a different polymerase performed better. This change in sensitivity due to a different polymerase was also noted by Sun et al. (2021).

The LAMP assay for *A. pleuropneumoniae* developed by Yang et al. (2009) lacked the desired sensitivity and was not colourimetric. The publication mentioned only that SYBER Green was used, but did not provide any information about its concentration. SYBER Green did not work well in the assay, and we switched to the WarmStart Colourimetric LAMP 2 x Master Mix (New England Biolabs, Massachusetts, USA), which produced a clear distinction between negative and positive results. We confirmed that the colour reaction results were due to amplification by running the amplification

products on a gel and performing PCRs simultaneously. There were no false results; the colour changes to yellow were always due to amplifications.
In the *E. coli* assay, the improvement in the limit of detection was due to the addition of guanidine chloride, which increased sensitivity by a factor of 10 and reduced amplification time. The addition of guanidine chloride to enhance sensitivity was suggested by Knox and Geddoe (2024), who used it to improve assay kinetics.

We set out to prove that LAMP assays could be used to test effluent for pathogenic bacteria. The ideal bacterium to demonstrate this was *Campylobacter coli*, due to its presence on pig farms worldwide and its high prevalence, such as approximately 72.7% in Australia (Owiredu et al., 2023). The first step was to demonstrate this using an established LAMP assay. We used the LAMP assay developed by Mason et al. (2020). We validated positive results with a PCR for *C. coli* (Wang et al., 2002). Neither assay was suitable for detecting *C. coli* in effluent in its current form. We had to not only develop an optimal extraction method but also had to optimise both assays. After developing an extraction method suitable for on-farm use and optimising the assays, we detected *C. coli* in effluent.

The second step was to show that *A. pleuropneumoniae* was detectable in effluent. This was done by PCR, as the LAMP assay was not established at this stage. Here again, we had to optimise the PCR first. We optimised the PCR for detecting *A. pleuropneumoniae* developed by Turni et al. (2014). After optimising the assay and applying the developed extraction method, we detected *A. pleuropneumoniae* in the effluent.

The last step of this project was to develop LAMP assays for *A. pleuropneumoniae* and *Escherichia coli*.
We ended up using the primers from an existing LAMP assay for *A. pleuropneuoniae*, but validated the assay with a different polymerase and used different buffers that came with the Master Mix.
For the *E. coli* assay, we started from scratch by designing 5 primer sets and testing these. The most promising variant was then used to develop the *E. coli* LAMP assay.
Both LAMP assays have proven to be highly sensitive, with sensitivities of $2.88 \times 10^3$ cfu/ml (2.88 cfu/µl) for *A. pleuropneumoniae* and $2.65 \times 10^2$ cfu/ml (0.265 cfu/µl) for *E. coli*. These sensitivity results fall within the declared sensitivity of 5 copies for LAMP assays by Craw and Balachandran (2012). Comparing this to conventional PCR and real-time PCR, the conventional PCR assays typically detect bacterial concentrations around $10^3$–$10^5$ cfu/mL (≈1–100 cfu/µL), whereas real-time quantitative PCR (qPCR) assays can detect $10^1$–$10^3$ cfu/mL (≈0.01–1 cfu/µL) depending on primer design, extraction efficiency and target gene copy number (Higgins et al., 2019) (Wang et al., 2019).

Regarding specificity, both assays were specific to their target genes: the *Apx* gene for *A. pleuropneumoniae* and the stb gene for *E. coli*.

In summary, the objectives of developing point-of-management LAMP assays that can detect respiratory and enteric bacterial pathogens in effluent have been achieved. This can serve as a platform for other bacterial pathogens, and the lessons learned on optimising the LAMP assay can be applied to developing other LAMP assays.

# 8 Implications & Recommendations

These LAMP assays can be used on farm, providing tools for monitoring bacteria in the effluent. This platform can be used for other bacterial species. For other pathogens, new primers have to be developed, and optimisation has to be done. However, the pathway to do this has been proven in this project, with the hardest part, the extraction of DNA, being done, which can be used for Gram-positive and Gram-negative bacteria.

There is a LAMP assay for *Pasteurella multocida*, that could be plugged into this platform.

These assays are easy to set up, but we would like to make this even easier; hence, we are contacting companies to explore the possibility of a company being interested in offering these tests as kits.

# 9 Intellectual Property

As these assays are a diagnostic tool, they might not be suitable for IP. In the past, it has not been regarded as a viable option for the diagnostic tests we have developed.

We will hand these assays to DPI to be added to their diagnostic tests for on-farm testing.

## 10 Technical Summary

We optimised a LAMP assay for *Campylobacter coli*, which was not very sensitive for *C. coli* in effluent. We optimised and designed part of a LAMP assay; the primers were previously published, but the assay was not sensitive and was not colourimetric. It had to be run on a gel to give results. We used the primers from this assay and changed everything else, making it colourimetric and more sensitive.

The *E. coli* assay we developed from scratch, including primer design and optimisation for extraction from effluent.

We also developed an extraction method that can be used on farm.

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## 12 Publications Arising

We are planning to write up the *A. pleuropneuomiae* and *E. coli* methods a manuscript.

## 13 Appendix

MICROBIAL GENOMICS

RESEARCH ARTICLE

Muralidhar *et al.*, *Microbial Genomics* 2025;11:001532

DOI 10.1099/mgen.0.001532

MICROBIOLOGY SOCIETY

OPEN DATA OPEN ACCESS

# Optimization of DNA extraction methods from pig farm wastewater for pathogen detection using metagenomic sequencing

Manavi Muralidhar[1,*], Muhammad Noman Naseem[1,†], Jose Ramon Botella Mesa[2], Lida Omaleki[1] and Conny Turni[1,*]

**Abstract**

Wastewater can be a useful sample to monitor disease outbreaks in the community, as it was demonstrated during the recent Severe acute respiratory syndrome coronavirus 2 pandemic. Due to housing conditions, diseases can rapidly spread within pig herds, resulting in high mortalities and significant economic losses. Monitoring piggery wastewater using Oxford Nanopore Technology's (ONT) sequencing platform combined with metagenomic analysis can provide early disease detection to deploy preventative measures. Nevertheless, obtaining DNA of the required purity and integrity from piggery wastewater is a major challenge. This study aims to identify and optimize the most effective method for obtaining high-quality and quantity DNA, which can be used in downstream applications for pathogen detection. Six DNA extraction protocols were tested on piggery wastewater samples and evaluated based on yield and overall DNA quality. The three best-performing methods, using commercially available kits (QIAGEN QIAamp® PowerFecal® Pro, QIAGEN DNeasy® PowerLyzer® PowerSoil® and Macherey-Nagel NucleoSpin® Soil), were then used to extract DNA from piggery wastewater samples spiked with a mock community composed of known pig pathogens. The extracted DNA samples were then sequenced on the ONT platform, and the effectiveness of the methods was evaluated using kraken2 taxonomic classifier and an in-house database. Results demonstrated that the optimized QIAGEN PowerFecal® Pro protocol was the most suitable and reliable extraction method. Overall, this study highlights the importance of determining the optimal DNA extraction method in effective disease surveillance using a complex environmental sample and takes an important step in making metagenomic disease surveillance a practical reality.

**Impact Statement**

Effective surveillance and early disease detection in livestock – critical not only for global food security but also for addressing the One Health challenges – are fundamental to mitigating outbreaks. Through this work, we evaluated six DNA extraction methods for their performance with piggery wastewater, revealing significant discrepancies in their ability to recover high-quality bacterial DNA from this complex matrix. Based on these findings, we identified three extraction kits that performed better and tested them for a further deeper analysis. By spiking piggery wastewater with a known mock community, we were able to demonstrate biases in DNA extraction processes, highlighting the variability that can influence pathogen detection. This work enabled us to select and optimize the most effective DNA extraction method for pathogen surveillance in piggery wastewater. From a broader perspective, our findings emphasize that to establish metagenomic surveillance as a routine tool, optimization and standardization of extraction methods for each matrix is critical to ensure reliable results that can drive early disease detection and intervention.

Link to the paper: **https://doi.org/10.1099/mgen.0.001532**