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Rapid on-farm diagnostics for bacterial respiratory disease outbreaks in poultry (RODBROP)

Rapid on-farm diagnostics for bacterial respiratory disease outbreaks in poultry (RODBROP)

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Project Summary

Project Title	Rapid on-farm diagnostics for bacterial respiratory disease outbreaks in poultry (RODBROP)
Project No.	21-308
Date	Start: 01/10/2021 End: 26/11/2023
Project Leader(s)	Seyed Ali Ghorashi
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Project Aim	The project aimed to develop and evaluate the efficacy of loop-mediated isothermal amplification (LAMP)-based assays for the precise and rapid detection of key avian respiratory pathogens, including *Pasteurella multocida*, *Mycoplasma gallisepticum*, and *Erysipelothrix rhusiopathiae*. In addition, the efficacy of different rapid DNA extraction procedures was also evaluated in conjunction with the developed LAMP assays, with the overall aim to facilitate the use of LAMP-based diagnostics at the point of care on-farm.
Background	The poultry industry is particularly vulnerable to infectious disease outbreaks as poultry farming tends to be intensive, and infectious disease can spread rapidly causing significant economic and welfare consequences. Early detection and intervention are crucial, but conventional diagnostics are time-consuming and expensive. LAMP-based diagnostic assays are well suited for on-farm deployment, as they do not require sophisticated laboratory equipment or specialised staff, yet can offer a diagnostic sensitivity and specificity that rivals conventional tests like PCR. However, LAMP-based assays, also rely on amplification of DNA fragments from target pathogens, and so require DNA templates extracted from clinical specimens. Therefore, while there are some available methods of field-based DNA extraction, this study aimed to evaluate their compatibility with colourimetric LAMP assays.
Research Outcome	Three specific LAMP-based diagnostic tests were developed for the detection of key poultry pathogens (*Pasteurella multocida, Mycoplasma gallisepticum, and Erysipelothrix rhusiopathiae*). Using clinical samples, the efficacy of these tests was demonstrated to be comparable to conventional tests like PCR.

Impacts and Outcomes	The key impact associated with this project is that it provides evidence indicating that routine cost-effective surveillance for key infectious pathogens in the poultry industry is possible. Three LAMP-based assays were developed, that can be performed without the need for sophisticated laboratory equipment, in conjunction with a rapid DNA extraction procedure in the field. In addition, diagnostic outcomes can be inferred based on simple visual observation of colour changes, which does not require any specialised expertise. Three standard operating procedures (SOPs) were developed for on-farm detection of the targeted poultry pathogens in this project. Three manuscripts have also been drafted that will be submitted for peer review in international level journals in the near future.
Publications	Comparative evaluation of PCR and loop-mediated isothermal amplification (LAMP) assays for detecting *Pasteurella multocida* in poultry. Madelaine Poussard, Sameer D. Pant, Jiongrui Huang, Peter Scott, Seyed Ali Ghorashi. Submitted to the New Zealand Veterinary Journal. Evaluation of a LAMP-based diagnostic assay targeting the *pvpa* gene for detection of *Mycoplasma gallisepticum* in poultry. Rebecca Mayne, Sameer Dinkar Pant, Amir Haji Noormohammadi, Jiongrui Huang, Peter Scott, Seyed Ali Ghorashi. In preparation. Development and evaluation of a colourimetric LAMP assay for detection of *Erysipelothrix rhusiopathiae* using a rapid DNA extraction procedure. Seyed Ali Ghorashi, Jiongrui Huang, Peter Scott, Sameer D. Pant. In preparation.

Executive Summary

This project aimed to design and develop three distinct loop-mediated isothermal amplification (LAMP) based diagnostic assays tailored for the detection of crucial avian respiratory pathogens or systemic infections: *Pasteurella multocida*, *Mycoplasma gallisepticum*, and *Erysipelothrix rhusiopathiae*. The project was executed sequentially in three phases, with each phase dedicated to the development of an individual LAMP assay. All three diagnostic tests were developed successfully, and a standard operating procedure (SOP) was developed and submitted alongside each milestone report, submitted upon the conclusion of each phase. Overall, the diagnostic tests developed as part of this project represent a significant advancement in avian health diagnostics, as they provide targeted tools for the rapid, reliable and specific identification of infectious pathogens crucial for the poultry industry. Infectious diseases are a major problem for the poultry industry as they spread rapidly on-farm. Early diagnosis and intervention are hampered by the unavailability of diagnostic tests that can be deployed on-farm. In this project, we have attempted to address this problem by developing rapid diagnostic tests for three key poultry infectious pathogens. In the first phase of the project, we developed a robust assay for the detection of *Pasteurella multocida*, a prominent respiratory pathogen in poultry. In the second phase, the project transitioned to the development of a LAMP assay tailored for the identification of *Mycoplasma gallisepticum*, another critical avian respiratory pathogen. In the final phase of the project, we optimised another LAMP-based assay specifically designed for the accurate detection of *Erysipelothrix rhusiopathiae* in poultry specimens. Moving forward, the developed LAMP assays could be implemented as routine diagnostic tools on poultry farms to enable rapid and specific identification of these targeted pathogens. A collaborative approach involving relevant stakeholders, veterinarians and poultry farms, is likely to facilitate assay adoption. Information and training programs may be needed to raise awareness and ensure proper usage by personnel. The assays should be continuously monitored and updated based on emerging pathogenic strains, and new assays targeting additional pathogens of economic importance may also be developed. It may also be possible to develop multiplex assays that allow for the simultaneous detection of multiple pathogens. A proactive approach emphasising routine surveillance is anticipated to enhance disease management, reduce economic losses, and contribute to the overall improvement of avian health in the poultry sector.

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Introduction

Respiratory diseases pose a significant threat to the health and productivity of animals in various agricultural sectors. Bacterial infections, particularly respiratory diseases in poultry, exhibit common clinical signs that make specific diagnosis difficult based solely on clinical observations. Among the causative agents, *Pasteurella multocida* (PM), *Mycoplasma gallisepticum* (MG), and *Erysipelothrix rhusiopathiae* (ER) are of particular concern due to their infectious nature. Current diagnostic procedures for these pathogens, including microbiological culture, serological testing, and PCR, are acknowledged for their accuracy but are hampered by their time-consuming and resource-intensive nature.

Pasteurella multocida, responsible for fowl cholera in poultry, demands precise and rapid diagnostic methods for effective management. Various diagnostic assays have been developed to detect PM, including microbiological culture (Kumar et al. 2004), serological testing (Heddleston et al. 1972), enzyme-linked immunosorbent assay (ELISA) (Marshall et al. 1981) and PCR (Townsend et al. 1998). Among these techniques, PCR has emerged as the most widely used diagnostic assay, and different PCR assays (targeting different genes) have been developed to detect PM strains (Dziva et al. 2008).

In addition, Mycoplasmosis frequently causes respiratory disease in avian species, and MG is a highly pathogenic strain that affects poultry health and productivity, by decreasing egg production in layers and the downgrading of carcasses in broilers (Feberwee et al. 2022). Moreover, MG can act as a predisposing factor for other respiratory diseases, such as Chronic Respiratory Disease (CRD), and often occurs concurrently with other pathogens like infectious bronchitis virus and *Avibacterium paragallinarum*, *Escherichia coli*, *Pasteurella multocida*, and various others (Chu & Uppal 1975; Wigle 2000). Traditional diagnostic methods, such as culture-based assays, have been the gold standard for detecting MG (Al-baqir et al. 2023), but they have two primary disadvantages. Firstly, culturing Mycoplasma can take several weeks, prolonging the turnaround time of culture based diagnoses, which in turn would delay the implementation of any control measures (Emam et al. 2020; Marouf et al. 2022). Secondly, culturing Mycoplasma requires specialised laboratory equipment and trained staff with the necessary expertise. On the other hand, serological tests offer a relatively rapid alternative for detection of MG, but these tests can sometimes cross-react with other mycoplasma strains (Feberwee et al. 2005; Kempf et al. 1997), leading to false positives and complicating the interpretation of results.

Erysipelothrix rhusiopathiae (ER), a rod-shaped and Gram-positive bacterium belonging to the phylum Firmicutes, is also a bacterium of significant concern in veterinary and agricultural settings due to its impact on animal and poultry health (Frana & Neubauer 2022). Erysipelas is a systemic and zoonotic disease caused by ER, which occurs globally as a septicemic infection affecting avian populations in addition to various other animal species, including swine, reptiles, amphibians and humans. Erysipelas can affect a wide range of poultry species, leading to significant mortality and egg production losses in affected flocks. The gross lesions observed in deceased birds during an outbreak are indicative of septicaemia (Eriksson 2019). Diagnosis usually requires a post-mortem, followed by the detection of ER in tissue samples through either bacterial culture or PCR (Clark 2015; Zhao et al. 2023). However, diagnostic methods currently available for ER are time-consuming and rely on specialised laboratory environments, making these test unsuitable for routine surveillance on-farm.

Traditional approaches such as PCR, though accurate, suffer from logistical challenges, requiring transportation of samples to specialised laboratories and posing contamination risks. This limitation underscores the urgent need for alternative diagnostics. Loop-mediated isothermal amplification

(LAMP) emerges as a promising solution, offering rapid, field-deployable testing capabilities with simplified equipment requirements and reduced contamination risks compared to PCR. While rapid DNA extraction methods do exist (Aithal et al. 2022; Truett et al. 2000; Zou et al. 2017), they have not yet been used in conjunction with colourimetric LAMP-based assays to develop on-farm diagnostic tests. Evaluating the sensitivity and specificity of LAMP-based assays in conjunction with these rapid DNA extraction procedures could be helpful in demonstrating the practicality and efficacy of LAMP technology for on-farm use.

In light of these challenges, our study explores and evaluates rapid DNA extraction methods compatible with LAMP for each bacterium, aiming to establish LAMP as a viable and efficient alternative for on-farm testing, providing rapid and accurate diagnostics in resource-limited settings.

Objectives

The primary goal of this study was to develop and assess a colourimetric LAMP assay designed for on-farm detection of PM, MG and ER outbreaks. The objectives were as below:

1. Development of a colourimetric LAMP assay:

Development of colourimetric LAMP assays specific to each bacterial strain for enhanced, rapid on-site detection.

2. Evaluation of rapid DNA extraction methods for field use:

Assessment of the efficacy of different rapid DNA extraction procedures tailored for on-farm application and evaluation of the compatibility of the rapid DNA extraction method with the developed LAMP assay.

3. Clinical sample testing:

Assessment of the applicability of the rapid DNA extraction method with a colourimetric LAMP assay in detecting bacterial presence in clinical samples. Evaluation of the performance of the developed assay in comparison to PCR for accurate and reliable detection in diverse clinical scenarios.

4. Determination of sensitivity, specificity, and accuracy of developed assays:

Evaluation of the sensitivity, specificity, and overall accuracy of the developed colourimetric LAMP assays and comparison of these performance metrics with those of PCR.

Methodology

Pasteurella multocida isolates

A total of ten *Pasteurella multocida* (PM) isolates/strains were utilised for development of a LAMP assay. Among these, seven isolates were obtained from the Veterinary Diagnostic Laboratory (VDL) at Charles Sturt University, cultured on blood agar plates. Additionally, two PM isolates were provided by the department of Avian Medicine at the University of Melbourne specifically for this research, and one PM vaccine strain was sourced from Bioproperties Pty Ltd. These samples were utilised as positive controls.

Mycoplasma gallisepticum isolates and clinical samples

Three distinct sample sets were used for the development and assessment of the LAMP assay. For assay development, the *Mycoplasma gallisepticum* (MG) vaccine strain (ts-11) was used as the positive control for both LAMP and PCR. Thirteen tracheal swabs were collected from 10 SPF chickens vaccinated with the ts-11 vaccine strain, and three unvaccinated chickens that were sourced from the University of Melbourne. The positive and negative controls for each experiment were the ts-11 MG vaccine strain and distilled water, respectively.

Erysipelothrix rhusiopathiae isolates

The development and evaluation of the LAMP assay involved three distinct sample sets. Firstly, the *Erysipelothrix rhusiopathiae* (ER) vaccine strain, Eryvac, was used as a positive control for both LAMP and PCR assay development. Twelve swab samples from various chicken organs were collected at a poultry farm experiencing erysipelas. These 12 clinical swabs underwent microbiological culturing before undergoing LAMP and PCR testing.

Additionally, 40 cloacal swabs were collected from turkeys for use as clinical samples in different diagnostic tests used in this study. The turkey flock, aged nine to 28 weeks, was clinically normal at the time the swabs were taken but had a history of ongoing fowl cholera, MG and ER outbreaks, and had not been vaccinated against PM, MG and ER or treated with antibiotics.

In addition to utilising positive controls and clinical samples, the specificity of each developed LAMP assay was assessed using a panel of diverse bacterial strains (Table 1). The bacterial strains were sourced from the Veterinary Diagnostic Laboratory (VDL) at Charles Sturt University. This set of bacterial species served as an evaluation tool to determine the specificity of each LAMP assay during its development phase.

Table 1 Bacterial strains used for testing specificity of the developed assays

	Bacterium
1	*Salmonella typhimurium*
2	*Streptococcus equi (subsp. zooepidemicus)*
3	*Staphylococcus aureus*
4	*Escherichia coli*
5	*Klebsiella pneumoniae*
6	*Staphylococcus sp.*
7	*Pseudomonas aeruginosa*
8	*Salmonella sp.*
9	*Enterobacter cloacae*
10	*Staphylococcus intermedius*
11	*Pasteurella multocida*
12	*Mycoplasma synoviae*
13	*Mycoplasma gallisepticum*
14	*Erysipelothrix rhusiopathiae*

DNA extraction methods

Two different DNA extraction methods were employed to extract DNA from PM isolates. The first method involved the use of Ly-14 Lysis Buffer which is used for SARS-CoV-2 Antigen testing (ACROBiosystems, USA). This is a rapid method for lysing the cells and involved the addition of clinical samples/swab to 150 µL of Ly-14 solution and incubating it at room temperature for 3–5 minutes. Two microlitres of this solution were used in the assay as a source of DNA.

The second method involved the use of a commercially available DNA extraction kit, i.e. Wizard® Genomic DNA Purification Kit (Promega, Australia), hereafter referred to as the commercial kit. The purpose of using this kit was to determine the efficacy of DNA extraction by using Ly-14 or any other rapid DNA extraction method by comparing the results with those obtained from the established commercial kit.

The DNA was extracted from all PM samples, including clinical samples using both methods, and to assess the quantity and quality of the extracted DNA, a NanoDrop spectrometer (Thermo Fisher Scientific, Australia) was employed.

Three DNA extraction methods were used for isolation of DNA from MG isolates. The first method involved the use of commercial kit. The second method involved the use of Ly-14 Lysis Buffer and the third method consisted of using Ly-14 buffer in combination with InstaGene Matrix (Chelex-100) (Cat No. 732-6030, Bio-Rad, Australia).

Each MG clinical sample was initially left to soak in the Ly-14 buffer for ten minutes, and subsequently squeezed against the sides of the tube to remove any Ly-14 buffer. At this stage, Ly-14 buffer was divided into two tubes (each ~250 µL). While the contents of first tube were directly used as DNA template, 100 µL Chelex-100 was added to the second tube, mixed and incubated at 56°C for 20 minutes before using the resulting solution as DNA template. Chelex-100 contains a resin that binds to inhibiting factors and prevents DNA degradation and was used in combination with Ly-14 buffer to improve the quality of extracted DNA. The swab was then used for DNA extraction using the commercial kit.

Two distinct DNA extraction methods were used to isolate DNA from ER specimens, both positive control (ER vaccine strain) and clinical samples. The first method involved the commercial kit, and DNA extraction was performed as per the manufacturer's instructions. The second method involved the Hot Sodium Hydroxide and Tris (HotSHOT) technique, initially developed by Truett et al. in 2000. The HotSHOT method provides a rapid and cost-efficient approach to genomic DNA extraction. This method involves the use of two primary solutions: a lysis solution comprised of 0.2 mM Di-Sodium EDTA and 25 mM NaOH with a pH of ~12, as well as a neutralising solution comprised of 40 mM Tris-HCl at a pH of ~1.9. In this method, the swab sample was soaked in 75 µl of the lysis solution, followed by an incubation period at 90°C for 10 minutes. Subsequently, an equal volume (75 µl) of the neutralising solution was added to the sample upon completion of the incubation. A two µl aliquot of this solution was used as template DNA in both ER-LAMP and ER-PCR assays. While the HotSHOT solution was initially designed for DNA extraction from mouse tissue, it has since been adapted to be used with a variety of different tissue samples, including clinical swab samples.

The PM, MG or ER vaccine, were each also diluted in dH_2O and one dose of each vaccine was added to a cotton swab, which was also individually subjected to DNA extraction using Ly-14 buffer, Ly-14 buffer plus Chelex-100, HotSHOT and the commercial kit extraction protocols, and were subsequently used as positive control DNA template in respective PCR and LAMP assays.

Primer design

All primers were designed using the Primer Explorer V5 software (Eiken Chemical Co., Tokyo, Japan).

The five PM LAMP primers including two outer primers (PM-F3 and PM-B3), two inner primers (PM-FIP and PM-BIP), and one loop primer (PM-LF) were designed based on the *Pasteurella multocida* subs *P. multocida* strain HN06 outer membrane protein (KMT1) gene (GenBank accession number CP003313).

The MG primers used for LAMP were designed based on the MG strain ts-11 *gapA* gene (GenBank accession number CP044225). The selection of the *gapA* gene was based on suitability, attributed to the lower variability of nucleotides among MG strains/isolates (Ghorashi et al. 2013). Five sets of primers targeting *gapA* were designed for MG.

The ER primers as published by Yamazaki et al. (2014), were employed in this study. These primers target a putative polypeptide gene associated with capsular polysaccharide synthesis and exhibit a high degree of conservation and specificity for ER.

To confirm the specificity for all primers, oligonucleotide sequences were analysed using the National Library of Medicine (NLM) basic local alignment search tool (BLAST®).

All specific primers designed for each individual LAMP assay were utilised in their respective reactions, with B3 and F3 primers specifically employed in the corresponding PCR assays for each target bacterium (Table 2). The colours in Table 2 correspond to the position of each primer in the target gene, as illustrated in Figure 1.

Table 2 Primer sequences utilised in PCR and LAMP assay

Primer Name	5'- 3' Primer Sequence
PM-F3	GGGCTTGTCGGTAGTCTT
PM-B3	AACGTAACTCAACATGGAAAT
PM-FIP	ATTGGCTCAACACACCAAACTCTTATTTGGCTTGTGGCAAAG
PM-BIP	TTGACAACGGCGCAACTGAT AGGAAATATAAACCGGCAAAT
PM-LF	GCCCAACAAAACTGTGCTTT
MG-F3	TCTAGAGCAACTAATGACTTCA
MG-B3	GACCTAAAGCTAATGCCAAG
MG-FIP	ACAAACACACTATTAGCTTGTGGATATTACCTCAAGTATTAGTTGATGG
MG-BIP	GTCTGACCAAGAATTCACTGGTTTGCAATTATGATGATCGGAATCG
MG-LB	TGATGCGCTTCCAGGTTATGT
ER-F3	ATCGTGGACCAAACAAGC
ER-B3	CTGATTCATGATCCCACATTG
ER-FIP	ACGTCGAACTGTTAGATATGCAAAAGATTTAACACAATTTTCAATCGTCC
ER-BIP	TTGTAATGATGAGCGCGATGAGGGAAAGATTCTCTTGATACTTTGA
ER-LF	CGATTGTCATAATCATCATAGCTC
ER-LB	CTTTTAGATTCTCAAGCCGCGTTA

The alignment of PM, MG and ER LAMP and PCR primers against the target DNA sequence is illustrated in Figure 1. All primers were ordered from Sigma Aldrich (Australia).

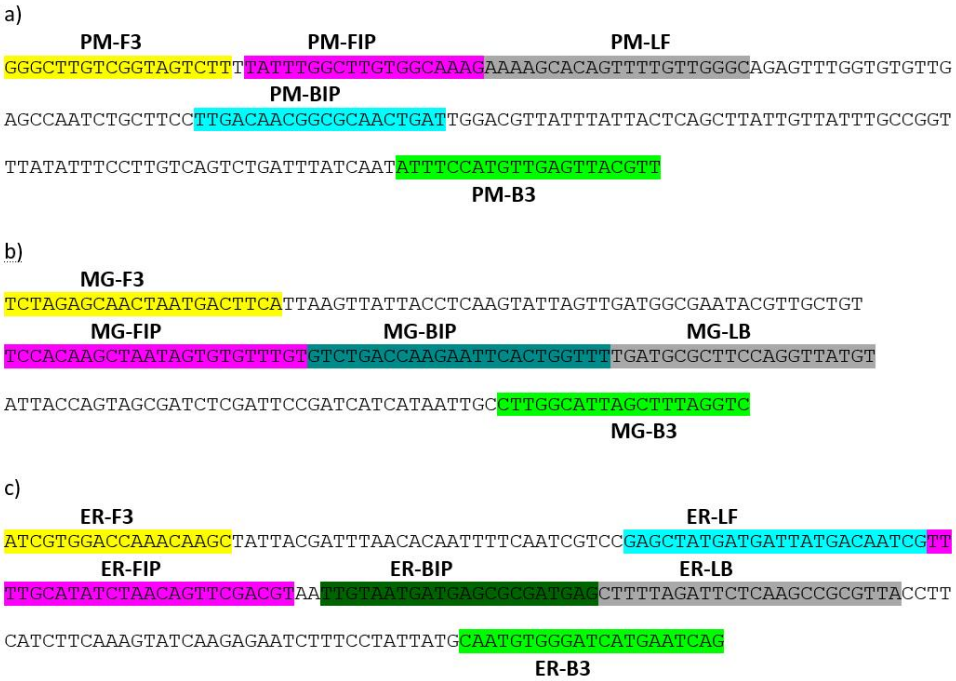

Figure 1 Positions of PCR (F3 and B3) and LAMP primers colour coded as per Table 2

a) KMT1 gene of *Pasteurella multocida* subs *p. multocida.*
b) gapA gene of *Mycoplasma gallisepticum.*
c) Putative polypeptide gene of *Erysipelothrix rhusiopathiae.*

PCR

To optimise the annealing temperature for each PCR, a gradient approach was employed and the optimal annealing temperatures were determined and used in all subsequent PCR experiments.

All positive control samples as well as clinical specimens were tested in PCR. The PCR amplification was performed in a 25 µL reaction volume. The reaction mixture consisted of 2 µL of extracted genomic DNA, 2 µM of each primer (F3 and B3), 1.5 mM $MgCl_2$, 100 µM of each dNTP, 5x GoTaq Green Flexi Reaction Buffer, and 1 U of GoTaq DNA Polymerase (Promega, Australia). The optimised PCR conditions for PM included an initial denaturation step at 95°C for 3 minutes, followed by 28 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR reaction was completed with a final extension step at 72°C for 5 minutes.

Thermocycling conditions for MG and ER PCRs were similar to that of PM except the annealing temperatures were 59°C and 55°C, respectively.

In each set of PCR assay, PM, MG or ER genomic DNA was included as a positive control, while distilled water was used as template in the negative control. To analyse the PCR products, samples were subjected to 1.5% agarose gel electrophoresis.

Loop-mediated isothermal amplification (LAMP)

A total volume of 20 µL was used for each LAMP reaction. This consisted of WarmStart Colourimetric LAMP 2X Master mix (New England Biolabs, Australia), the LAMP primers: inner primers (FIP and BIP) at 16 µM each, outer primers (F3 and B3) at 2 µM each, and loop primers (LF and BF) at 4 µM and 2 µL of template DNA. This reaction was then incubated at 65°C for 60 minutes in a thermal cycler. The successful amplification of each bacterial (PM, MG or ER) DNA was determined by observing a colour change from red to yellow in the tested samples.

DNA sequencing

PCR amplicons obtained from each positive control (from three different vaccine samples, i.e. PM, ts-11 and Eryvac) were subjected to bidirectional sequencing using F3 and B3 primers, carried out by Australian Genome Research Facility Ltd (AGRF Ltd., Brisbane).

The sequence data was further analysed using ClustalW software (Thompson et al. 1994) and BioEdit Sequence Alignment Editor (version 6.0.9.0) for multiple sequence alignments. Subsequently, if there were multiple samples sequenced, a phylogenetic tree was constructed to analyse and illustrate the genetic relationships between each sample.

Specificity and limit of detection of developed assays

The specificity of both PCR and LAMP assays for each bacterial strain were evaluated by extracting DNA from a panel of non-related bacterial species (Table 1), which were subsequently tested using PCR and LAMP. DNA was extracted using the commercial kit. To assess the limit of detection, 10-fold dilutions of 1 ng/µL DNA extracted from each vaccine strain were prepared and serially diluted, and each dilution was tested in PCR and LAMP, respectively.

Comparison of PCR and LAMP as detection methods

While bacterial culture remains the gold standard for definitive identification of each bacterial strain (PM, MG and ER), molecular techniques such as PCR have gained popularity due to their heightened

sensitivity, faster turnaround time, and the ability to detect the bacterium even in cases with low bacterial loads. In order to facilitate a comparative analysis between the two methods, PCR was designated as the gold standard, and the sensitivity and specificity of each LAMP assay were evaluated against their respective PCR counterparts. This assessment was conducted using the MEDCALC 2 x 2 contingency table available at www.medcalc.org/calc/diagnostic_test.php.

Results

The results for development of a LAMP assay for each bacterial strain, and comparison with PCR are distinctly outlined under their respective bacterial strain titles. This approach ensures a focused and organised presentation of findings specific to *Pasteurella multocida*, *Mycoplasma gallisepticum*, and *Erysipelothrix rhusiopathiae*, enhancing the clarity and accessibility of the results for each targeted pathogen.

Comparative analysis of LAMP and PCR techniques for *Pasteurella multocida*

The target gene sequence of *Pasteurella multocida* was subjected to BlastN analysis against the NCBI Nucleotide collection (nr/nt) database, which contains 500 entries of different isolates of *P. multocida*. The BlastN analysis yielded hits with 100% sequence coverage and more than 98% identity, confirming the accuracy and reliability of the target gene sequence.

In addition, in silico analysis using BlastN reaffirmed the specificity of the designed primers, suggesting a low likelihood of false positive or false negative results in the PCR or LAMP assays. This indicates that the primers are highly specific to the target gene and are expected to provide accurate and reliable results in the molecular assays.

Detection of *Pasteurella multocida* using PCR and LAMP assay

Prior to the testing of clinical samples, all *P. multocida* isolates/strains including the vaccine strain were tested. Following PCR amplification, agarose gel electrophoresis was performed on all tested specimens. The gel analysis revealed the presence of a single DNA fragment of the expected size, approximately 200 base pairs, in all *P. multocida* isolates/strains. All *P. multocida* isolates and vaccine strain were also positive in LAMP (Figure 2).

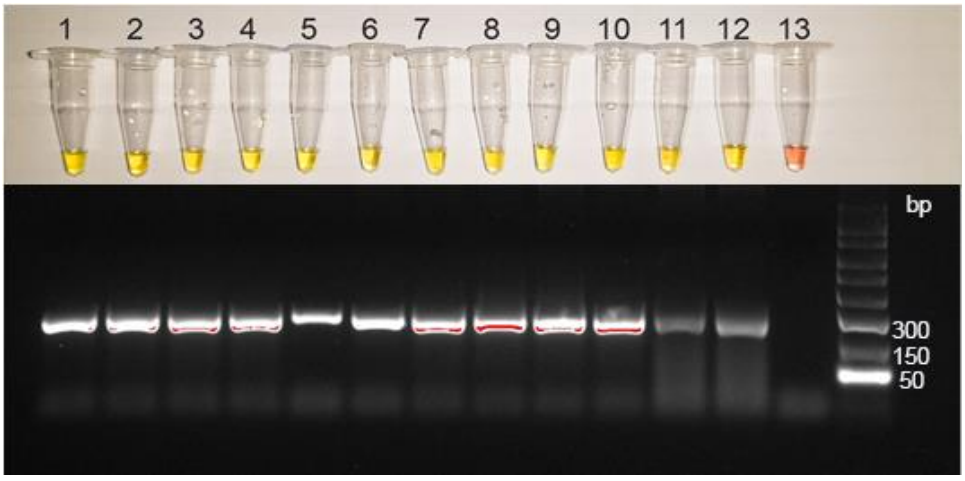

Figure 2 Detection of *P. multocida* in positive samples by LAMP and PCR with gel electrophoresis

Lane 1: PM-9-VDL, Lane 2: PM-10-VDL, Lane 3: PM-11-VDL, Lane 4: PM-12-VDL, Lane 5: PM-15-VDL, Lane 6: PM533191, Lane 7: PM1103918, Lane 8: PMP181111A (PM vaccine), Lane 9: PM22-189-1, Lane 10: PM22-189-2, Lane 11: PM 22-189-1 (Ly-14), Lane 12: PM22-189-2 (Ly-14), Lane 13: Negative control.

Specificity and limit of detection for PCR and LAMP

The specificity of PCR and LAMP assays was evaluated by testing a panel of non-related bacterial species. Both PCR and LAMP assay did not yield positive results when used in conjunction with unrelated bacteria (Table 3).

Table 3 Evaluation of non-related bacterial strains: Specificity outcomes for *Pasteurella multocida* obtained from PCR and LAMP analyses

Bacterium	LAMP	PCR
Salmonella typhimurium	-	-
Streptococcus equi (subsp. zooepidemicus)	-	-
Staphylococcus aureus	-	-
Escherichia coli	-	-
Klebsiella pneumoniae	-	-
Staphylococcus sp.	-	-
Pseudomonas aeruginosa	-	-
Salmonella sp.	-	-
Enterobacter cloacae	-	-
Staphylococcus intermedius	-	-
Mycoplasma gallisepticum	-	-
Mycoplasma synoviae	-	-
Erysipelothrix rhusiopathiae	-	-
Pasteurella multocida	+	+
Negative control	-	-

+ a positive detection.
- a negative result.

The limit of detection for each assay was inferred using serial 10-fold dilutions of DNA extracted from *P. multocida* vaccine strain. Serial dilutions were made using 1 ng/µL to 10^{-6} ng/µL of DNA. PCR and LAMP both produced a positive result up to 10^{-2} ng/µL dilution (Figure 3).

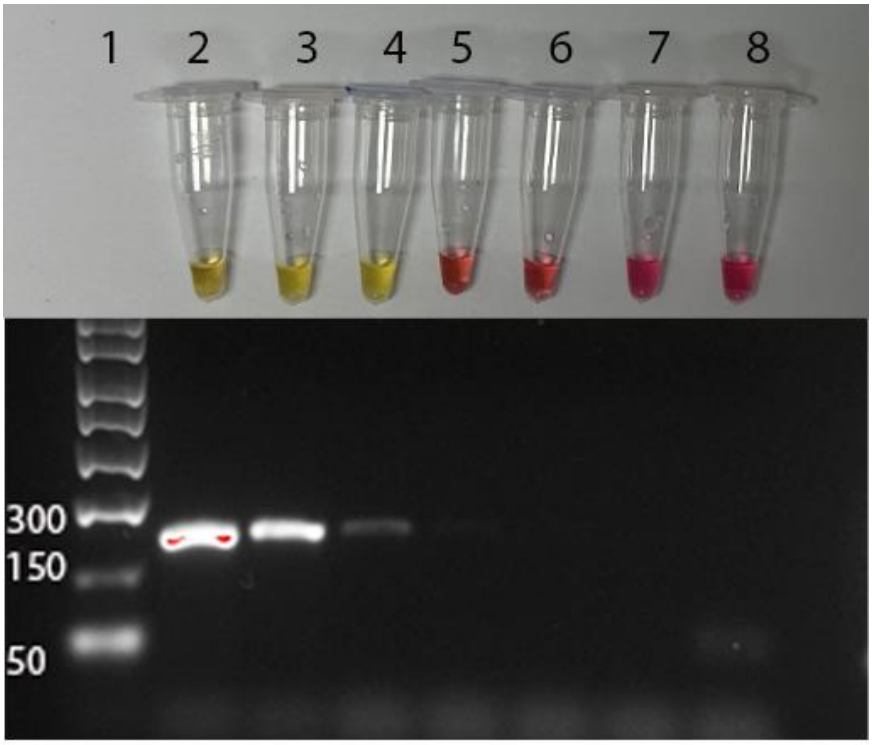

Figure 3 Limit of detection of PCR and LAMP at different dilutions of *P. multocida* DNA

Lane 1: Molecular marker (Sigma-Aldrich, Australia).
Lanes 2–8 corresponding with the DNA concentration 1ng, 10^{-1} ng, 10^{-2} ng, 10^{-3} ng, 10^{-4} ng, 10^{-5} ng and 10^{-6} ng, respectively.

Detection of *Pasteurella multocida* in clinical samples using PCR and LAMP assay

Forty cloacal swabs were collected as clinical samples from a flock of turkeys, and subjected to clinical testing. DNA from these samples were extracted using two different extraction methods (Ly-14 and the commercial kit), and all samples were tested via both PCR and LAMP assays. DNA samples extracted by Ly-14 buffer were all negative when tested by PCR and LAMP. Out of 40 DNA samples extracted by the commercial kit, two were positive in PCR (samples 33 and 37) and four were positive in LAMP (samples 32, 33, 37 and 40). The results are shown in Table 4.

Table 4 Results of testing clinical samples in PCR and LAMP using two different extraction methods

Sample number/DNA extraction method	PCR		LAMP	
	Ly-14	Promega	Ly-14	Promega
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
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27	-	-	-	-
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-
31	-	-	-	-
32	-	-	-	+
33	-	+	-	+
34	-	-	-	-
35	-	-	-	-
36	-	-	-	-
37	-	+	-	+
38	-	-	-	-
39	-	-	-	-
40	-	-	-	+

DNA sequencing

All *P. multocida* isolates were subjected to DNA extraction and subsequent sequencing, and the resulting sequences were aligned to generate a DNA sequence alignment (Figure 4).

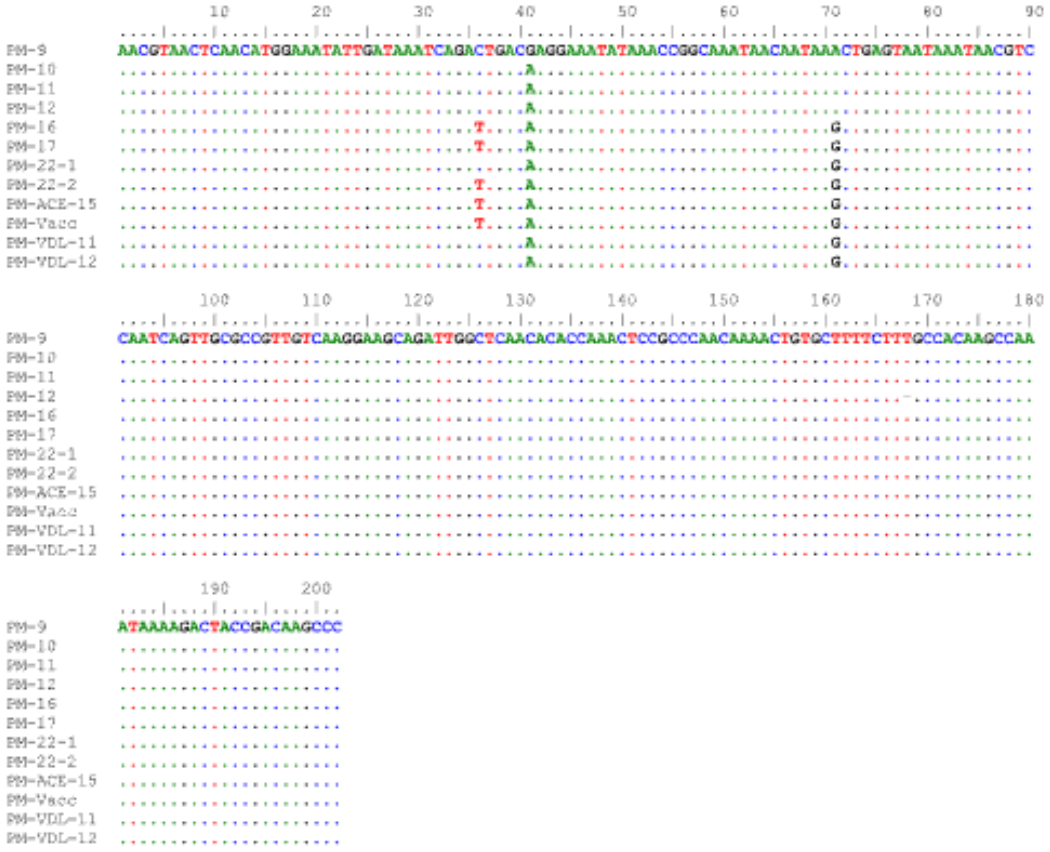

Figure 4 DNA sequence alignment of PCR amplicons for *P. multocida* samples

Identical nucleotides are shown by "."

Furthermore, a phylogenetic tree was constructed to infer the genetic relationships among the samples using the Neighbour-Joining method (Saitou & Nei 1987) (Figure 5).

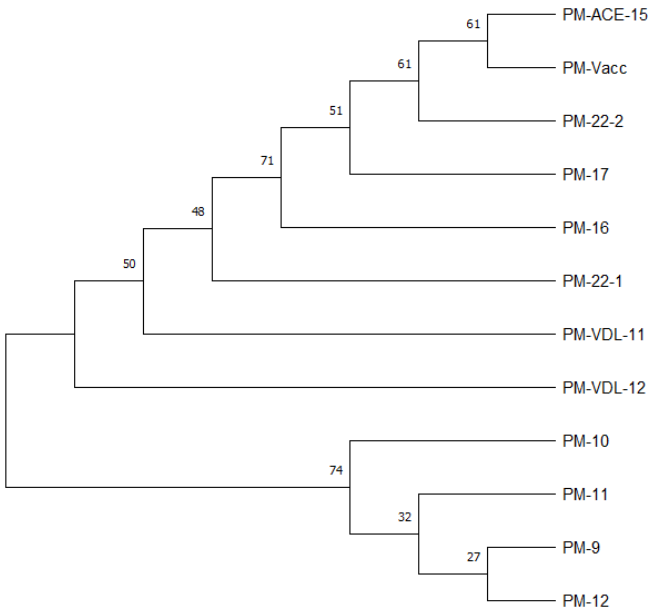

Figure 5 Phylogenetic tree showing the evolutionary relationships between the *P. multocida* samples

Comparison of specificity and sensitivity of PCR and LAMP assay

The specificity and sensitivity of both PCR and LAMP assays were analysed using a 2 x 2 contingency table (Ghorashi et al. 2022). The PCR was considered as the gold standard test and LAMP results were compared against PCR. The analytical sensitivity and specificity assessment was performed using DNA extracted (using the commercial kit) from vaccine strain of *P. multocida,* and from unrelated bacterial colonies (pure bacterial cultures) (Table 1). These DNA samples were relatively pure with minimal inhibitory factors. Analytical sensitivity and the specificity for PCR and LAMP assays evaluated by 2 x 2 contingency table was calculated to be 100%.

Clinical sensitivity and specificity were assessed using DNA from clinical samples (cloacal swabs), which were tested in both assays using the two DNA extraction methods. The sensitivity of LAMP was identical to PCR (100%) but the specificity of LAMP was found to be slightly lower (94.7%) compared to PCR (100%), which was due to two false positives identified in the LAMP assay (Table 5).

Table 5 Clinical sensitivity and specificity for PCR and LAMP assay evaluated by 2 x 2 contingency table

Clinical sensitivity/specificity	PCR	LAMP
Sensitivity	100%	100%
Specificity	100%	94.7%
Accuracy	100%	95%

Given the limited number of clinical samples tested (40), further testing with a larger and more diverse sample set is warranted to account for variability in sample composition, potential rare events, and to calculate statistical confidence intervals, ensuring a more robust assessment of assay accuracy and confirming the reported accuracy rates conclusively.

At the conclusion of this section, readers are referred to Appendices 1 and 2, where a detailed standard operating procedure (SOP) for performing the rapid DNA extraction using LY-14 and PM LAMP assay discussed in this segment are provided.

Comparative analysis of LAMP and PCR techniques for *Mycoplasma gallisepticum*

Detection of *Mycoplasma gallisepticum* using PCR and LAMP

For MG positive controls, a PCR using the B3 and F3 primers successfully amplified the expected DNA fragment of approximately 197 bp, as observed through agarose gel electrophoresis. Conversely, PCRs involving a negative control did not display any visible bands on agarose gel electrophoresis. Similarly, in the LAMP assays, negative controls maintained an unchanged reaction colour (red), while the use of MG positive controls resulted in a positive test outcome (yellow colour change), indicative of successful amplification of the target DNA (Figure 6). MG isolates testing with PCR targeting *16S rRNA* gene also produced an expected DNA fragment of around 303 bp. Results from testing tracheal swabs collected from 10 chickens vaccinated with the MG ts-11 strain and three unvaccinated chickens showed that both PCRs and LAMP could only detect MG in one vaccinated bird using all three DNA extraction methods (Table 6).

Table 6 PCR and LAMP results for testing tracheal swabs

Swabs from vaccinated/unvaccinated bird/DNA extraction methods	**LAMP**			**PCR (F3+B3)**			**PCR (*16S rRNA*)**		
	Ly-14	**Ly-14+Chelex100**	**Commercial kit**	**Ly-14**	**Ly-14+Chelex100**	**Commercial kit**	**Ly-14**	**Ly-14+Chelex100**	**Commercial kit**
vaccinated	-	-	-	-	-	-	-	-	-
vaccinated	-	-	-	-	-	-	-	-	-
vaccinated	-	-	-	-	-	-	-	-	-
vaccinated	-	-	-	-	-	-	-	-	-
vaccinated	-	-	-	-	-	-	-	-	-
vaccinated	-	-	-	-	-	-	-	-	-
vaccinated	-	-	-	-	-	-	-	-	-
vaccinated	-	-	-	-	-	-	-	-	-
vaccinated	+	+	+	+	+	+	+	+	+
vaccinated	-	-	-	-	-	-	-	-	-
unvaccinated	-	-	-	-	-	-	-	-	-
unvaccinated	-	-	-	-	-	-	-	-	-
unvaccinated	-	-	-	-	-	-	-	-	-
Positive control	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	-	-	-	-

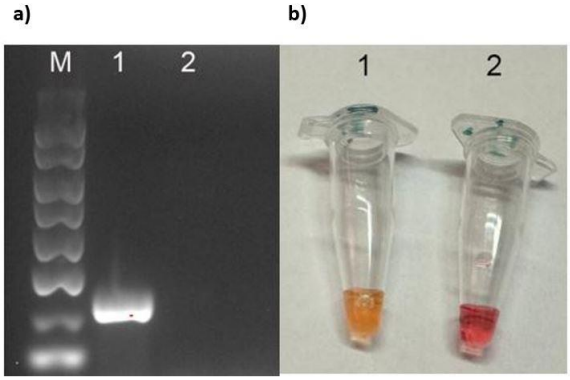

Figure 6 MG PCR and MG LAMP assay results

a) MG PCR M: DNA marker, Lane 1: Positive control (ts-11 vaccine strain), Lane 2: Negative control.
b) MG LAMP assay Tube 1: Positive control (ts-11 vaccine strain), Tube 2: Negative control.

The specificity of each test was evaluated using DNA from 13 different bacterial strains in each assay. Both PCR and LAMP assays detected MG and did not produce positive results for unrelated bacterial strains (Table 7).

Table 7 Assessment of unrelated bacterial strains: PCR and LAMP specificity results for *Mycoplasma gallisepticum*

Bacterium	LAMP	PCR
Salmonella typhimurium	-	-
Streptococcus equi (subsp. zooepidemicus)	-	-
Staphylococcus aureus	-	-
Escherichia coli	-	-
Klebsiella pneumoniae	-	-
Staphylococcus sp.	-	-
Pseudomonas aeruginosa	-	-
Salmonella sp.	-	-
Enterobacter cloacae	-	-
Staphylococcus intermedius	-	-
Pasteurella multocida	-	-
Mycoplasma synoviae	-	-
Erysipelothrix rhusiopathiae	-	-
Mycoplasma gallisepticum	+	+
Negative control	-	-

+ a positive detection.
- a negative result.

Detection of *Mycoplasma gallisepticum* in clinical samples using PCR and LAMP assay

Forty cloacal swabs collected from a flock of turkeys were tested. DNA from these samples were extracted using three different extraction methods (Ly-14, Ly-14 + Chelex100 and a commercial kit) and all samples were subjected to both PCR and LAMP assays. Only two DNA samples extracted by Ly-14 buffer tested positive for MG via PCR (samples 27 and 28) and three samples tested positive via LAMP (samples 27, 28 and 34). The same results were obtained for both PCR and LAMP when DNA samples were extracted using Ly-14 + Chelex100. On the other hand, when DNA samples were

extracted from the 40 samples via the commercial kit, two were positive in PCR (samples 27 and 39) and only one was positive when tested by LAMP (sample 27). Results are shown in Table 8.

Table 8 Cloacal swabs tested by PCR (F3 and B3 primers) and LAMP using 3 different DNA extraction methods

DNA extraction	Ly-14		Ly-14+Chelex100		Commercial kit	
Cloacal swab	LAMP	PCR	LAMP	PCR	LAMP	PCR
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	-	-	-	-	-	-
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-
19	-	-	-	-	-	-
20	-	-	-	-	-	-
21	-	-	-	-	-	-
22	-	-	-	-	-	-
23	-	-	-	-	-	-
24	-	-	-	-	-	-
25	-	-	-	-	-	-
26	-	-	-	-	-	-
27	+	+	+	+	+	+
28	+	+	+	+	-	-
29	-	-	-	-	-	-
30	-	-	-	-	-	-
31	-	-	-	-	-	-
32	-	-	-	-	-	-
33	-	-	-	-	-	-
34	+	-	+	-	-	-
35	-	-	-	-	-	-
36	-	-	-	-	-	-
37	-	-	-	-	-	-
38	-	-	-	-	-	-
39	-	-	-	-	-	+
40	-	-	-	-	-	-
Positive control	+	+	+	+	+	+
Negative control	-	-	-	-	-	-

Detection limit and specificity of PCR and LAMP

The limit of detection for PCR, utilising serial 10-fold dilutions of DNA extracted from the MG vaccine strain, was estimated to be around 10^{-3} ng, whereas the sensitivity of LAMP was estimated to be around 10^{-2} ng (Figure 7). This indicates that PCR is approximately 10 times more sensitive than LAMP when DNA was extracted using the commercial kit.

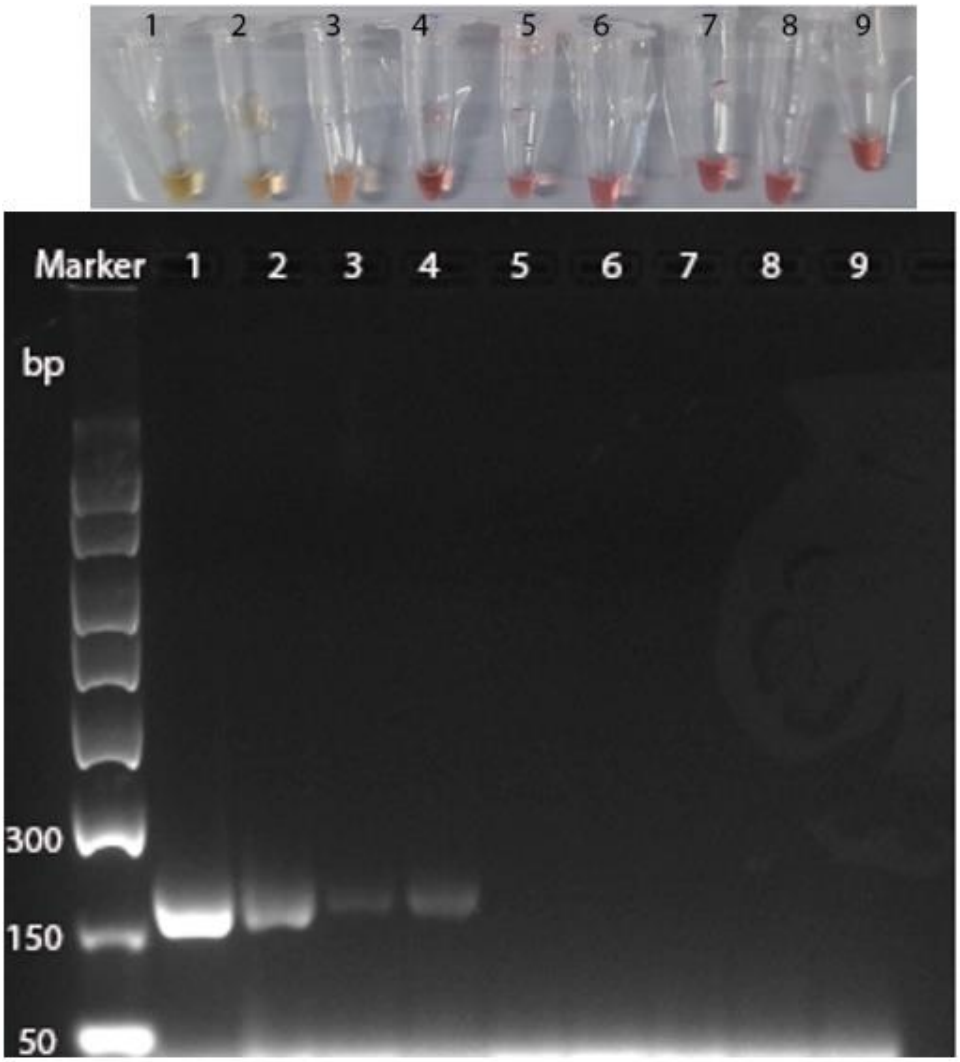

Figure 7 Detection limit of PCR and LAMP using serial dilutions of MG DNA

Lane 1: 1ng of DNA, Lane 2: 10^{-1} ng, Lane 3: 10^{-2} ng, Lane 4: 10^{-3} ng, Lane 5: 10^{-4} ng, Lane 6: 10^{-5} ng, Lane 7: 10^{-6} ng, Lane 8: 10^{-7} ng, Lane 9: negative control.

Comparison of analytical specificity and sensitivity of PCR and LAMP assay

Analytical specificity and sensitivity of both PCR and LAMP assay were compared using a 2 x 2 contingency table. PCR performed using DNA extracted via the commercial kit, was considered the gold standard, and LAMP results were compared against PCR outcomes. Evaluation of analytical sensitivity and specificity involved DNA extracted from the MG vaccine strain and various bacterial strains from unrelated cultured colonies, ensuring relatively pure samples with minimal inhibitory factors. Both PCR and LAMP exhibited 100% sensitivity and specificity (Table 9).

Table 9 Analytical sensitivity and specificity for PCR and LAMP assay using 13 unrelated bacterial strains evaluated by 2 x 2 contingency table

Laboratory sensitivity/specificity	PCR	LAMP
Sensitivity	100%	100%
Specificity	100%	100%
Accuracy	100%	100%

Comparison of clinical specificity and sensitivity

Clinical sensitivity and specificity were assessed using DNA extracted from experimental (tracheal swabs from vaccinated and unvaccinated chickens) and clinical samples (40 cloacal swabs), which were all subjected to both PCR and LAMP.

Table 6 presents PCR and LAMP results obtained by testing tracheal swabs collected from vaccinated/unvaccinated birds using different DNA extraction methods. The results from PCR and LAMP assays were in agreement with each other, and this agreement was consistent across the different DNA extraction procedures used. Considering PCR with DNA extracted from a commercial kit as the gold standard, LAMP produced consistent results compared to both PCR assays (using the F3+B3, and MG1273f + MG1427r primers). The sensitivity and specificity of both PCR and LAMP, assessed using tracheal swabs, were found to be 100%.

Testing 40 cloacal samples showed comparable results between LAMP and PCR when Ly-14 and Ly-14+Chelex100 were used for DNA extraction. However, results from PCR and LAMP using DNA extracted via the commercial kit showed a slight difference, with PCR detecting sample 39 as positive while LAMP did not (Table 8). Sample 34 tested positive in LAMP when DNA was extracted using Ly-14 and Ly-14 + Chelex100, whereas it tested negative in all PCR tests, even when DNA was extracted using Ly-14 and Ly-14 + Chelex100. Additionally, when sample 34 was extracted with a commercial kit, LAMP yielded a negative result. Since the results from PCR using DNA extracted with the commercial kit were used as the gold standard test, the positive result of LAMP for sample 34 was interpreted as a false positive result in LAMP, possibly due to the presence of impurities in the rapid DNA extraction method. The sensitivity, specificity, and accuracy of LAMP for detecting MG in cloacal samples were 50%, 100%, and 97.5%, respectively, when DNA was extracted via a commercial kit. However, when Ly-14 and Ly14+Chelex100 were used for DNA extraction, the sensitivity, specificity, and accuracy of LAMP in detecting MG in cloacal swabs were calculated to be 100%, 97.4%, and 97.5%, respectively (Table 9).

Table 9 The sensitivity, specificity, and accuracy of PCR and LAMP for detecting MG in tracheal and cloacal samples using three DNA extraction methods

Samples	Tracheal swabs		Cloacal swabs					
DNA extraction method	Ly-14, Ly-14+Chelex100 and commercial kit		Ly-14		Ly-14+Chelex100		Promega kit	
	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR
Sensitivity %	100	100	100	100	100	100	50	100
Specificity %	100	100	97.4	100	97.4	100	100	100
Accuracy %	100	100	97.5	100	97.5	100	97.5	100

DNA sequencing

The sequencing results showed identical nucleotide sequences between MG ts-11 and positive specimens, including one from a vaccinated and three from clinical samples (samples 27, 28, and 39). Confirmatory specificity for MG amplification was achieved through nucleotide sequence alignment using the NCBI Blast tool.

In conclusion of this section, readers are directed to Appendix 3, where detailed SOP for conducting the MG LAMP assay discussed in this segment is available.

Comparative analysis of LAMP and PCR techniques for *Erysipelothrix rhusiopathiae*

Detection of *Erysipelothrix rhusiopathiae* using PCR and LAMP

When subjected to PCR, the ER positive control yielded a DNA amplicon of the expected size (207 bp) in agarose gel electrophoresis, while the negative control did not yield any DNA bands. Similarly, in LAMP assay, the negative control yielded a negative result (with no change in reaction colour, i.e. remaining red), whereas the ER positive control produced a positive result (indicated by a colour change to yellow, indicating the amplification of the target DNA) (Figure 8).

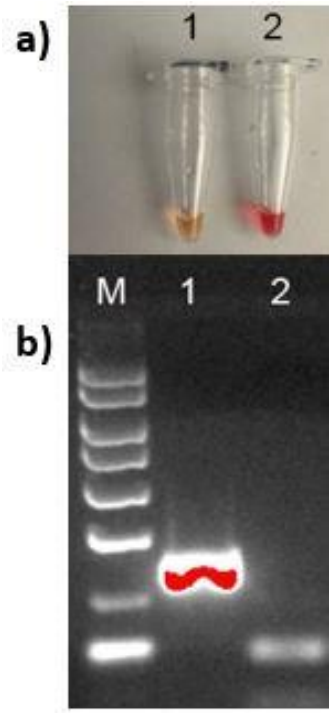

Figure 8 ER PCR and ER LAMP assay results

a) ER LAMP assay, Tube 1: Positive control (ER vaccine strain), Tube 2: Negative control.
b) ER PCR, M: DNA marker, Lane 1: Positive control (ER vaccine strain), Lane 2: Negative control.

The specificity of PCR and LAMP were assessed by subjecting them to DNA from 13 distinct bacterial strains. In both assays, only ER was detected, with no positive results observed for unrelated bacterial strains. The outcomes of the specificity test are presented in Table 10.

Table 10 PCR and LAMP results for assessment of unrelated bacterial strains

Bacterium	LAMP	PCR
Salmonella typhimurium	-	-
Streptococcus equi (subsp. zooepidemicus)	-	-
Staphylococcus aureus	-	-
Escherichia coli	-	-
Klebsiella pneumoniae	-	-
Staphylococcus sp.	-	-
Pseudomonas aeruginosa	-	-
Salmonella sp.	-	-
Enterobacter cloacae	-	-
Staphylococcus intermedius	-	-
Pasteurella multocida	-	-
Mycoplasma synoviae	-	-
Mycoplasma gallisepticum	-	-
Erysipelothrix rhusiopathiae	+	+
Negative control	-	-

+ a positive detection.
- a negative result.

The limit of detection for PCR and LAMP

PCR and LAMP assays performed using serial ten-fold dilutions of DNA extracted from the vaccine strain, indicated that both were capable of detecting as little as 10^{-3} ng of extracted DNA in the reaction mixture (Figure 9).

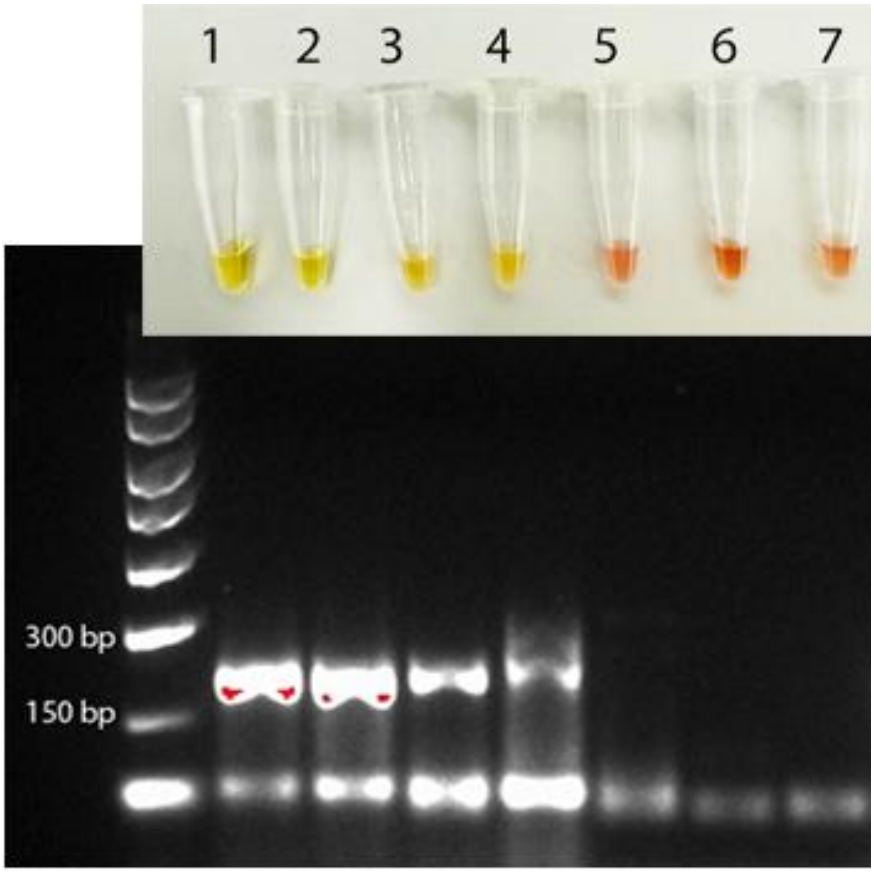

Figure 9 Detection limit of PCR and LAMP using serial 10 fold dilutions of DNA template

Lane 1: 1ng of DNA, Lane 2: 10^{-1} ng, Lane 3: 10^{-2} ng, Lane 4: 10^{-3} ng, Lane 5: 10^{-4} ng, Lane 6: 10^{-5} ng and Lane 7: 10^{-6} ng of DNA.

Detection of *Erysipelothrix rhusiopathiae* in clinical samples using PCR and LAMP assay

Both PCR and LAMP assays were successful in detecting ER in liver, spleen, and bone marrow samples collected from chickens suspected to be infected in the field. The same positive outcome was observed for the positive control, which consisted of the ER vaccine strain. Conversely, the negative control (dH_2O) produced consistently negative results in both PCR and LAMP assays (Figure 10).

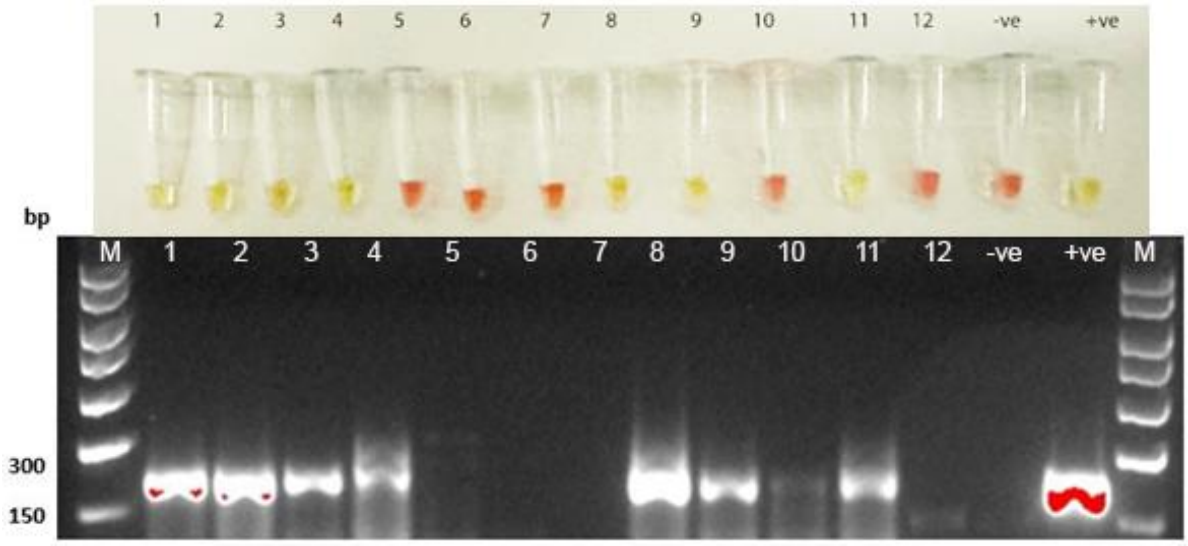

Figure 10 LAMP and PCR results for 12 clinical swabs from different organs collected from infected chickens

M: DNA marker, Lane 1: Liver, Lane 2: Bone marrow, Lane 3: Liver, Lane 4: Spleen, Lane 5: Bone marrow, Lane 6: Bone marrow, Lane 7: Bone marrow, Lane 8: Liver, Lane 9: Liver, Lane 10: Bone marrow, Lane 11: Spleen, Lane 12: Bone marrow, -ve: Negative control and +ve: Positive control (ER vaccine strain).

The detection outcomes are further compared with control samples, using two different DNA extraction procedures, the commercial kit and the HotSHOT method. The results indicated that samples 1, 3, 4, 8, 9, and 11, originating from the liver and spleen, consistently produced positive results in both PCR and LAMP assays, indicating the presence of ER. However, samples 5, 6, 7, 10 and 12, which were bone marrow samples, produced negative results in culture and LAMP assay when DNA was extracted using a commercial kit or the HotSHOT method, indicating the absence of the pathogen in these particular samples, except for a single instance in PCR where sample 10 DNA extracted using the commercial kit produced a faint but positive band on agarose gel electrophoresis. The results for positive and negative controls confirm the effectiveness of both PCR and LAMP assays, and demonstrate their reliability in identifying ER in the test.

Table 11 presents a summary of the results obtained from PCR and LAMP assays for clinical organ samples collected from infected chickens with potential *Erysipelothrix* spp. infections.

Table 11 PCR and LAMP results for clinical organ samples collected from infected chickens for the presence of *Erysipelothrix spp.*, as well as control samples using two DNA extraction methods

Sample No.	Source	Bacterial culture	PCR		LAMP	
			Commercial kit	HotSHOT	Commercial kit	HotSHOT
1	Liver	+	+	+	+	+
2	Bone marrow	+	+	+	+	+
3	Liver	+	+	+	+	+
4	Spleen	+	+	+	+	+
5	Bone marrow	-	-	-	-	-
6	Bone marrow	-	-	-	-	-
7	Bone marrow	-	-	-	-	-
8	Liver	+	+	+	+	+
9	Liver	+	+	+	+	+
10	Bone marrow	-	+	-	-	-
11	Spleen	+	+	+	+	+
12	Bone marrow	-	-	-	-	-
Positive control	ER vaccine strain	NT	+	+	+	+
Negative control	dH_2O	NT	-	-	-	-

+ a positive detection.
- a negative result.
NT Not tested.

The results from testing clinical swabs from different organs indicated that these molecular tests can effectively detect ER in different organ samples, especially when testing bone marrow samples with high fat content.

Given that all clinical organ samples were subjected to DNA extraction using the two distinct methods, HotSHOT and commercial kit methods, it is noteworthy that the results obtained from both DNA preparations consistently demonstrated similarity in both PCR and LAMP assays, resulting in nearly identical outcomes except for sample 10 using commercial DNA extraction kit and PCR. If the bacteriological culture is considered as the gold standard test, this PCR result may be regarded a false positive.

A total of 40 cloacal samples were also collected from a turkey flock and were subjected to DNA extraction using both the HotSHOT and commercial kit methods, and PCR and LAMP assays were conducted on these DNA samples (Table 12). All 40 samples showed negative results in both the PCR and LAMP assays, except for the positive control, which effectively confirms the absence of ER in the collected samples (Table 12).

Table 12 Cloacal samples were analysed through PCR and LAMP, using two DNA extraction techniques

DNA extraction technique	HotSHOT		Commercial kit	
Cloacal swab	LAMP	PCR	LAMP	PCR
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-
18	-	-	-	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	-	-	-
24	-	-	-	-
25	-	-	-	-
26	-	-	-	-
27	-	-	-	-
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-
31	-	-	-	-
32	-	-	-	-
33	-	-	-	-
34	-	-	-	-
35	-	-	-	-
36	-	-	-	-
37	-	-	-	-
38	-	-	-	-
39	-	-	-	-
40	-	-	-	-
Positive control (ER vaccine strain)	+	+	+	+
Negative control	-	-	-	-

Comparative analysis of PCR and LAMP specificity and sensitivity

Analytical sensitivity and specificity assessments were conducted using DNA samples extracted from the ER vaccine strain and a diverse selection of bacterial cultures obtained from unrelated strains. Given that these samples, consisting of bacterial cultures, demonstrated minimal presence of inhibitory factors, both PCR and LAMP assays exhibited robust performance. Specifically, both assays successfully detected ER in the samples from the vaccine strain and produced negative results when tested with unrelated bacterial isolates. Therefore, the assays demonstrated 100% analytical sensitivity and specificity in this controlled experimental setting.

DNA sequencing

The sequencing results of the ER PCR product revealed a 100% identical match to the ER strain when subjected to NCBI Blast, confirming the specificity of the amplification.

Evaluation of clinical sensitivity and specificity for PCR and LAMP assay

The performance of PCR and LAMP tests in detecting ER in 52 clinical samples including swabs from various organs and cloacal swabs was assessed using a 2 x 2 contingency table. Two DNA extraction methods, HotSHOT and the commercial kit, were used in conjunction with each sample. Microbiological culture was considered the gold standard for detecting the pathogen. The results indicate that both PCR and LAMP tests showed 100% clinical sensitivity in all scenarios, regardless of the DNA extraction method used. PCR demonstrated a specificity of 97.78% when using the commercial kit, while LAMP maintained a 100% specificity in all cases. The accuracy of PCR in detecting ER in clinical samples was slightly lower (98.08%) when compared with LAMP assay (100%) (Table 13). Overall, these results demonstrate that both PCR and LAMP are able to accurately detect ER in clinical samples.

Table 13 Comparison of clinical sensitivity and specificity for PCR and LAMP assay using different DNA extraction methods via 2x2 contingency table

	Bacterial culture	**PCR**		**LAMP**	
DNA extraction method	NA	**Commercial kit**	**HotSHOT**	**Commercial kit**	**HotSHOT**
Sensitivity %	100	100	100	100	100
Specificity %	100	97.78	100	100	100
Accuracy %	100	98.08	100	100	100

At the end of this section, readers are referred to Appendices 4 and 5, which contain comprehensive SOPs for performing the HotSHOT DNA extraction and ER LAMP assay discussed in this section.

Discussion

This study primarily aimed to develop three field-deployable LAMP-based assays for detecting *Pasteurella multocida* (PM), *Mycoplasma gallisepticum* (MG) and *Erysipelothrix rhusiopathiae* (ER), in conjunction with a rapid DNA extraction procedure that can be used in field without needing any sophisticated laboratory equipment. The isothermal nature of LAMP-based assays negates the need for thermal cycling, which makes these assays suited for use in field. However, despite being described for the first time in 2000, the use of LAMP-based diagnostics in field conditions has been restricted, in part, due to the lack of associated field-based template preparation methods and product detection formats (Njiru 2012). Therefore, in addition to developing each LAMP assay, the study also aimed to evaluate its efficacy against a conventional diagnostic test like PCR, when applied to clinical samples in conjunction of a rapid field based DNA extraction procedure.

In this section, individualised analyses of the results for PM, MG and ER will be presented under dedicated subheadings. This structured approach enables a focused examination of the unique findings associated with each bacterial strain, specifically exploring implications of the LAMP and PCR techniques. Subsequently, a comprehensive discussion will integrate insights from all three bacterial strains, offering a broader perspective on shared patterns and implications.

Pasteurella multocida

Pasteurella multocida, causing fowl cholera, poses economic threats to Australian poultry, particularly in free range systems systems (Blackall et al. 2020; Singh et al. 2013; Singh et al. 2014). The increased cases stem from wildlife exposure, emphasising the need for a diagnostic test detecting diverse serotypes (Blackall et al. 2020; Singh et al. 2013). While PCR, targeting the KMT1 gene, is accurate but cumbersome, LAMP presents a faster, simpler, and potentially portable alternative, ideal for on-farm deployment, saving time and costs (Townsend et al. 2001). This shift addresses challenges in routine surveillance, early detection, and containment of disease outbreaks in poultry populations.

The study compared LAMP and PCR assays for detecting *P. multocida*. LAMP showed comparable efficacy with 100% sensitivity and 94.74% specificity. While PCR had slightly higher specificity (100%), LAMP offered faster results in 90 minutes, opposed to PCR's 180 minutes, saving time and avoiding transportation costs. LAMP, without gel electrophoresis, minimised contamination risk, and its immediate colour change in positive samples facilitated easy interpretation, especially suitable for point-of-care testing (Wong et al. 2020).

The study aimed to evaluate Ly-14 Lysis buffer for field DNA extraction in portable LAMP for *P. multocida* detection. While Ly-14 worked well with pure bacterial cultures, its use with clinical samples, likely contaminated with inhibitory substances like faecal material, hindered PCR and LAMP efficiency (Alaeddini 2012). The Ly-14 lysate, used in both assays, possibly inhibited PCR due to its high protein concentration (Schrader et al. 2012; Sidstedt et al. 2020). Further research exploring alternative clinical sample-specific DNA extraction methods is needed to enhance LAMP accuracy and reliability in field applications, urging additional testing with diverse clinical samples for optimisation.

LAMP's sensitivity varies in studies; some find it less sensitive than PCR (Garner et al. 2022; Ghorashi et al. 2022), while others report comparable or higher sensitivity (Bhimani et al. 2015a; Sun et al. 2010). This variability may stem from diverse LAMP protocols, with researchers adjusting primer design and conditions for improved sensitivity (Chaouch 2021). Additionally, the choice of detection method, like fluorescent dyes, affects sensitivity (Zhao et al. 2020), highlighting the need for standardised protocols in evaluating LAMP performance.

In our experiments, both PCR and LAMP showed comparable sensitivities, detecting as little as 10 pg of DNA. This seems inconsistent with studies suggesting LAMP's higher sensitivity for *P. multocida* (Bhimani et al. 2015a). Sensitivity's context-dependent nature emphasises the need for meticulous consideration of specific factors in comparisons, such as primer sequences, reaction conditions, and DNA extraction methods. Bhimani et al. (2015) reported a significantly lower limit of detection for LAMP (22.8 pg/µl) than PCR (2.28 ng/µl), indicating LAMP's potential to detect *P. multocida* DNA at concentrations 10 times lower than PCR. These variations underscore the importance of standardised protocols and parameter selection based on study objectives and resources, explaining the observed sensitivity similarity in our study.

Mycoplasma gallisepticum

Diagnosing *Mycoplasma gallisepticum* (MG) in the poultry industry is challenging due to the lack of distinctive clinical features. Conventional culture-based diagnostics are specific but time-consuming and require specialised equipment, hindering on-farm deployment. This limits routine surveillance and early outbreak detection crucial for effective poultry disease management (Feberwee et al. 2005; Kempf et al. 1997; Salisch et al. 1998). Serological tests like ELISA provide quicker diagnoses without culturing specimens, but cross-reactivity and delayed detection can limit efficacy (Feberwee et al. 2005; Kempf et al. 1997). Molecular assays like PCR offer high sensitivity but require sophisticated equipment and controlled labs, hindering on-farm deployment. LAMP assays, not dependent on specialised equipment or personnel, offer on-farm potential for MG surveillance. Their isothermal nature, coupled with simple DNA extraction using Ly-14, eliminates complex protocols and equipment needs.

The Ly-14 method of DNA extraction (97.4% sensitivity) is comparable to a commercial kit (100%) in MG detection. This suggests feasibility for on-site MG detection using LAMP assays with Ly-14 for rapid, efficient results within 80 minutes. In assessing the analytical sensitivity and specificity of both PCR and LAMP assays, we utilised established methods. This included testing 10-fold dilutions of MG DNA for sensitivity evaluation and assessing specificity using 13 unrelated bacterial species. These approaches align with previously validated protocols (Kursa et al. 2015; Zhang et al. 2015).LAMP and PCR both demonstrated 100% sensitivity and specificity in MG detection from tracheal swabs in a controlled lab. However, with cloacal swabs simulating real-world conditions, LAMP's specificity decreased to 97.4% using Ly-14 extraction, while PCR maintained 100%. Commercial kits resolved false positives in LAMP. Careful sample handling, especially during DNA extraction, is crucial due to LAMP's susceptibility to false positives in the presence of contaminants.

Field-collected samples, possibly contaminated with faecal material, may contribute to false positives in LAMP assays. Contaminants and the use of 4–6 primers, differentiating LAMP from PCR, pose challenges. The complexity of LAMP reactions and potential unintended cross-reactivity among multiple primers underscore the need for specificity in primer design to minimise false positives, as highlighted in other studies (Hardinge & Murray 2019; Huang et al. 2022). Consideration of sample conditions and careful primer design is crucial for reliable LAMP results in field applications.

Erysipelothrix rhusiopathiae

A field-ready LAMP-based assay for *Erysipelothrix rhusiopathiae* (ER) detection, paired with a rapid on-site DNA extraction procedure, eliminated the need for sophisticated laboratory equipment. LAMP's isothermal nature makes it suitable for field use, yet its application has been limited due to the absence of associated field-ready template preparation methods. The study not only developed

the LAMP assay but also assessed its efficacy against PCR, using a rapid on-site DNA extraction method for clinical samples.

LAMP-based diagnosis showed accuracy (100%) equal to traditional culture and superior to PCR for detecting ER in clinical samples. The rapid DNA extraction method, paired with the LAMP assay, effectively captured DNA. Clinical specimens often have minimal DNA, and avoiding loss during extraction is crucial for accurate results. The procedure didn't require specialised lab equipment, making it farm-deployable for routine surveillance (Truett et al. 2000). This highlights its potential in on-site diagnostics for cost-effective and efficient monitoring.

Our findings also indicate that LAMP-based assays can provide cost-effective alternatives to conventional laboratory-based diagnostics like PCR. In colourimetric LAMPs in particular, the ability to infer results by simple visual observations of a colour change, negates the need for any additional expenses unlike PCR where agarose gel electrophoresis is frequently needed to visualise successful DNA amplification and infer results.

Furthermore, in terms of costs involved in routine surveillance and diagnosis, the need for a cold chain represents a considerable expense during the transportation and storage of molecular diagnostic reagents (Njiru 2012). For instance, transporting these reagents from diagnostic laboratory to the field requires the use of dry ice and incurs substantial transportation charges. This still applies to LAMP-based assays, and therefore the viability of lyophilising LAMP reagents needs to be explored in future to eliminate the need for a cold chain during transportation and storage, which would further improve the utility of LAMP based assays in diverse settings, providing a valuable advantage in scenarios where maintaining a constant cold chain might be logistically challenging or financially burdensome (Thekisoe et al. 2009). This study demonstrates the efficacy, cost-effectiveness, and potential for on-farm deployment of a field-ready LAMP assay for detection of ER, while also addressing challenges related to field application.

Conclusion

Advancements in on-site diagnostics for poultry pathogens, as demonstrated through LAMP assays, present promising alternatives to traditional methods. The studies on *Pasteurella multocida*, *Mycoplasma gallisepticum*, and *Erysipelothrix rhusiopathiae* showed the efficacy, cost-effectiveness, and potential for on-farm deployment of LAMP-based assays. Despite challenges, such as false positives and variability in sensitivity, these assays offer rapid results, simplicity, and reduced dependency on specialised equipment, making them valuable tools for early disease detection and management in the poultry industry. Future research should focus on standardisation, optimisation, and addressing specific challenges to enhance the reliability and applicability of LAMP-based diagnostics in diverse settings.

Implications

This study demonstrates that LAMP-based diagnostic assays perform comparably to conventional diagnostic tests like PCR in detecting three key poultry bacterial pathogens. Results indicate that while conventional PCR performed in a laboratory setting affords accurate and reliable diagnosis, LAMP-based diagnostics can afford rapid turn-around times, simplicity, and portability, making these tests well-suited for use on-farm for routine surveillance of infectious diseases. Development of a rapid and portable DNA extraction method could pave the way for point-of-care testing for poultry pathogens in the field. Given the economic significance of respiratory pathogens, rapid and accurate diagnosis is essential for effective disease management in the poultry industry. In addition to the immediate advantages of rapid turn-around times and simplicity, the potential portability of the LAMP assay, coupled with the development of a rapid and portable DNA extraction method, could create novel opportunities for on-farm diagnosis in the poultry industry. On-farm diagnostic tests have the potential not only to streamline and enhance poultry health management but also to contribute significantly to the overall resilience and sustainability of the poultry industry.

Recommendations

The recommendations for the Australian poultry industry arising from the project are as follows:

1. Invest in LAMP-based diagnostics: We recommend investing in LAMP-based diagnostics for routine surveillance of key pathogens in poultry farms. Our project provides evidence that demonstrates the efficiency of LAMP assays in promptly and accurately identifying avian respiratory pathogens, presenting a valuable tool for early disease detection. Our research team at CSU is willing to work collaboratively with PHA to help with the development and adoption of LAMP-based assays in routine testing procedures, and to facilitate the integration of LAMP technology into industry practices.
2. Training programs: We recommend the implementation of training programs for poultry industry professionals such as veterinarians, servicemen and farmers in the use of LAMP assays. While LAMP-based assays are user-friendly and do not require extensive expertise, training programs are essential to encourage adoption by industry personnel to conduct on-site pathogen detection, enhancing overall biosecurity measures. The research team at CSU that developed these tests would be willing to support such initiatives.
3. Collaboration with research institutions on the development of rapid diagnostics: To address emerging poultry pathogens, industry stakeholders are encouraged to collaborate with research institutions. This partnership can contribute to the ongoing development of new LAMP assays, expanding the versatility of this technology in poultry disease management. Moreover, the current research team at CSU is open to collaboration with other institutions, offering its expertise and resources to collectively advance the development of rapid diagnostics for the poultry industry. Utilising CSU's state-of-the-art facilities, extensive expertise, and hands-on experience in LAMP assay development, can contribute to expanding the application of LAMP assays for various significant poultry pathogens.
4. Exploring lyophilisation of diagnostic reagents for elimination of cold chain requirements: We recommend future investigations should explore the possibility of lyophilising LAMP reagents, aiming to eliminate the necessity for a cold chain during transportation and storage. This research avenue promises to significantly enhance the practicality of LAMP-based assays, especially in scenarios where maintaining a constant cold chain poses logistical or financial challenges.

Media and Publications

For each phase of the project, including the development of each LAMP assay and DNA extraction procedure, a standard operating procedure (SOP) was meticulously prepared and submitted. In addition, three manuscripts, each corresponding to the development of the individual LAMP assays, are currently in preparation. These manuscripts aim to provide comprehensive details on the methodologies, findings, and applications of the developed LAMP assays for the detection of *Pasteurella multocida*, *Mycoplasma gallisepticum*, and *Erysipelothrix rhusiopathiae*. The anticipated completion and submission dates for these manuscripts are expected in 2024. Upon completion, PHA will be notified, seeking permission for manuscript submission to facilitate widespread dissemination of the project outcomes.

Intellectual Property or Confidential Information Arising

N/A

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Appendices

Appendix 1

DNA EXTRACTION USING LY-14

PURPOSE

This SOP describes the procedure for field extraction of DNA from clinical samples, bacterial cultures and vaccines using a lysis buffer.

SCOPE

Using this method, DNA can be extracted from a variety of sample types, including dry (swab), and liquid (vaccine or culture) specimens. The procedure is crude as it is designed to be used in the field without the need for specialised laboratory equipment, and involves lysis of cells contained in any clinical specimen, releasing DNA contained within these cells. Lysed samples containing DNA can then be used in molecular tests such as PCR or LAMP assays to detect specific target sequences for the pathogen of interest. Extracted DNA from some clinical samples (cloacal swabs, etc.) may have relatively high levels of contaminants and should be used cautiously. This method is suitable for respiratory swabs, vaccine or culture specimens.

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https://www.acrobiosystems.com/P4274-Lysis-Buffer-for-SARS-CoV-2-Antigen-Test-Development.html

SAFETY

The appropriate personal protective equipment **(e.g. gloves and laboratory coat)** must be worn when handling samples, DNA extraction reagents and products.

MATERIALS

- Autoclaved 1.5 ml tubes
- Pipette (200 µl -10000 µl), calibrated
- Pipette tips (200 µl and 1000 µl), autoclave sterilised
- Ly-14 buffer (Focus Bioscience, Cat. No. Ly-14-10 ml)

PROCEDURE

Collection of specimens and processing

- Dry cotton swabs taken from respiratory secretions or gross lesions, or isolated bacteria can be used for DNA extraction.
- A positive swab and one sterile swab should also be used and processed similarly as positive and negative control, respectively.
- Set up and label one microfuge tube (1.5 ml) for each sample plus two tubes for positive and negative controls.
- Add 150 µl Ly-14 solution to each 1.5 ml tube.

- Insert the swab sample into the 1.5 ml lysis tube. Rotate the swab five times and leave it in the solution for three minutes.
- Remove swab, and drain liquid from the swab as much as possible by pressing the swab against the edges of the tube.
- Dispose of the swab appropriately.
- Use the solution as the source of DNA template in LAMP reaction or PCR.

WASTE DISPOSAL

All waste material is to be placed in the laboratory biological waste bins, and then autoclave sterilised prior to disposal.

Appendix 2

LAMP DIAGNOSTIC FOR THE DETECTION OF *Pasteurella multocida* (Fowl cholera)

PURPOSE

Describes the procedure for performing a colourimetric loop-mediated isothermal amplification (LAMP) assay for the detection of *Pasteurella multocida* in swabs taken from suspect chickens.

SCOPE

The causative agent of fowl cholera (FC) is *Pasteurella multocida* (*P. multocida*), a gram-negative bacterium that can affect all birds including chickens, turkeys and wild birds. The disease caused by this bacterium in birds is also known as avian cholera, avian haemorrhagic septicaemia or avian pasteurellosis. Fowl cholera is a contagious disease and clinical signs depend on the course of the disease. Fowl cholera can cause mortality without clinical signs in its acute form and can lead to swollen wattles, lameness and respiratory infection in its chronic form. Effective control of FC is essential to minimise disease associated economic losses and requires early detection of the disease. While isolation of bacteria via microbiological culturing can be used to identify *P. multocida*, this method is time consuming and less sensitive than molecular diagnostics. Molecular diagnostics based on PCR and LAMP assays are alternative detection methods, and LAMP assay has the advantage that it requires less time to be completed as well as being suitable to be performed on-farm without requiring sophisticated laboratory equipment (Bhimani et al. 2015b; Glisson 2013). The LAMP assay is an alternative to bacterial culture and is a useful diagnostic tool for the detection of *P. multocida*.

REFERENCES

- Appendix 1 – DNA extraction method
- Bhimani, M., Bhanderi, B., & Roy, A. (2015). Loop-mediated Isothermal Amplification assay (LAMP) based detection of *Pasteurella multocida* in cases of haemorrhagic septicaemia and fowl cholera. *Vet Ital*, *51*(2), 115-121. https://doi.org/10.12834/VetIt.242.812.4
- Glisson, J. R., Hofacre,Charles L., Christensen, Jens P. (2013). *Fowl Cholera*. In D. E. Swayne, J.R. Glisson, L.R. McDougald, L.K. Nolan, D.L. Suarez, V.L. Nair, (Ed.), *Diseases of Poultry* (13 ed., pp. 658-676). Wiley-Blackwell.

SAFETY

The appropriate personal protective equipment **(e.g. gloves and laboratory coat)** must be worn when handling samples, LAMP reagents and related products.

PRIMER DETAILS

Primer	Sequence (5'-3')
PM-F3	GGGCTTGTCGGTAGTCTT
PM-B3	AACGTAACTCAACATGGAAAT
PM-FIP	ATTGGCTCAACACACCAAACTCTTATTTGGCTTGTGGCAAAG
PM-BIP	TTGACAACGGCGCAACTGATAGGAAATATAAACCGGCAAAT
PM-LF	GCCCAACAAAACTGTGCTTTT

MATERIALS

- Autoclaved PCR tubes (0.2 ml) are used for LAMP assay
- Pipette (1-10 µl), calibrated
- Pipette tips (10 µl), autoclave sterilised
- ddH_2O
- WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs, Cat. No. M1800S or M1800L)
- 10X LAMP primer mix (Sigma-Aldrich, custom desalted 50 nmol)
- Crushed ice and esky or cold block
- Heat block to provide 65°C temperature

PROCEDURE

Collection of specimens

- Dry cotton swabs taken from liver or respiratory secretions such as nasal discharge; or even isolated bacteria can be used as clinical specimens for field DNA extraction.
- In conjunction with each clinical specimen, a swab known to be positive, and another sterile swab should also be used and processed similarly as positive and negative control, respectively.

DNA extraction protocol

Please see Appendix 1 for instructions.

Primer Mix

- The LAMP assay requires the use of five primers. Lyophilised primer sets can be diluted to 100 µM stock solutions by resuspending in nuclease free dH_2O, for long term storage in any ordinary freezer (-20°C).
- Prepare a 10X working stock primer mix as per the specified volumes in the table below (can be kept at -20°C).

Primer	**10X concentration (Stock)**	**Volume (µl)**
PM-F3	2 µM	2
PM-B3	2 µM	2
PM-FIP	16 µM	16
PM-BIP	16 µM	16
PM-LF	4 µM	4
ddH_2O	-	60
Total volume (Mix primers)		100

Master Mix

- Identify the number of clinical specimens to be tested, then add a positive control and a negative control to each batch of clinical specimens. This constitutes the number of LAMP tests required.
- **NB:** The LAMP tubes should be labelled and set up accordingly prior to preparing the reaction mix.
- Set up each LAMP reaction by combining the reagents noted in the table below, in the specified quantities.
- Flick (to mix gently) the sample and add 2 µL to the appropriate LAMP tube.
- Use positive and negative swabs in your DNA extraction along with test swabs. NB: Positive control DNA extracted from *Pasteurella multocida* vaccine strain could also be used.
- For the negative control, add 2 µL of Ly-14 buffer.

Add each reagent to the tube in the order mentioned in the table below and keep the LAMP tubes on ice or a cold block during preparation of the reaction.

Reagent	Quantity (µL)
WarmStart Colorimetric LAMP 2X Master Mix	10.0
10X primer mix	2.0
dH2O	8.0
Extracted DNA	0.0
Total volume per reaction	20.0

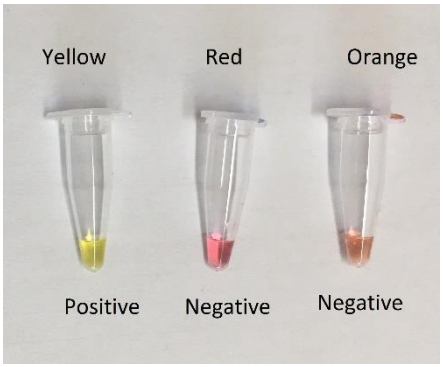
Run LAMP Test

- Set up the temperature at 65°C in a heat block.
- Place the LAMP tubes at 65°C for 1 hour.
- Upon completion of the incubation, remove the reaction tubes and interpret the results as below:

  Yellow colour = Positive

  Red colour = Negative

  Orange colour = Negative



The test is invalid if the reaction colour in positive control is red/orange and/or the reaction colour in negative control is yellow.

Appendix 3

LAMP DIAGNOSTIC FOR THE DETECTION OF *Mycoplasma gallisepticum*

PURPOSE

Describes the procedure for preparing and running a colourimetric loop-mediated isothermal amplification (LAMP) assay for the detection of *Mycoplasma gallisepticum* (MG) in swabs taken from suspect chickens.

SCOPE

Mycoplasma gallisepticum is a contagious bacterial disease that affects poultry, especially chickens, and can cause significant economic losses in the poultry industry. The disease is characterised by respiratory symptoms, such as coughing, sneezing, and nasal discharge, and can lead to decreased egg production and increased mortality rates (Armour 2020). The culturing method for diagnosis of *Mycoplasma gallisepticum* is not commonly used because this method is often tedious and time-consuming. It can take a few weeks for the bacteria to grow and form colonies in the culture medium, which can delay the diagnosis and treatment of infected flocks. Since early detection of *Mycoplasma gallisepticum* is crucial to prevent the spread of the disease and minimise economic losses, a rapid diagnostic assay such as loop-mediated isothermal amplification (LAMP) can provide quick and accurate results in the field (Ehtisham-Ul-Haque et al. 2017). This technology can detect the presence of the bacteria in a sample within 90 minutes, enabling early intervention to prevent the spread of the disease and protect the health and welfare of poultry flocks.

REFERENCES

- Appendix 1 – DNA Extraction method
- Ehtisham-Ul-Haque, S., Kiran, M., Waheed, U., & Younus, M. (2017). Real-time Loop-mediated Isothermal Amplification (LAMP) of mgc2 Gene of *Mycoplasma gallisepticum*. J Vet Res, 61(4), 439-444. https://doi.org/10.1515/jvetres-2017-0058
- Armour, N. K. (2020). *Mycoplasma gallisepticum* Infection. In D. E. Swayne (Ed.), Diseases of Poultry (14 ed., Vol. 1, pp. 911-923). Wiley-Blackwell.

SAFETY

The appropriate personal protective equipment **(gloves and laboratory coat)** must be worn when handling samples, LAMP reagents and products.

PRIMER DETAILS

Primer	**Sequence (5'-3')**
MG-F3	TCTAGAGCAACTAATGACTTCA
MG-B3	GACCTAAAGCTAATGCCAAG
MG-FIP	ACAAACACACTATTAGCTTGTGGATATTACCTCAAGTATTAGTTGATGG
MG-BIP	GTCTGACCAAGAATTCACTGGTTTGCAATTATGATGATCGGAATCG
MG-LB	TGATGCGCTTCCAGGTTATGT

MATERIALS

- Autoclaved PCR tubes (0.2 ml) are used for LAMP assay
- Pipette (1-10 µl), calibrated
- Pipette tips (10 µl), autoclave sterilised
- ddH_2O
- WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs, Cat. No. M1800S or M1800L)
- 10X LAMP primer mix (Sigma-Aldrich, custom desalted 50 nmol)
- Crushed ice and esky or cold block
- Heat block to provide 65°C temperature

PROCEDURE

Collection of specimens

- Dry cotton swabs taken from liver or respiratory secretions such as nasal discharge; or even isolated bacteria can be used as clinical specimens for field DNA extraction.
- In conjunction with each clinical specimen, a swab known to be positive, and another sterile swab should also be used and processed similarly as positive and negative control, respectively.

DNA extraction protocol

- Please see Appendix 1 for instructions.

Primer Mix

- The LAMP assay requires the use of five primers. Lyophilised primer sets can be diluted to 100 µM stock solutions by resuspending in nuclease free ddH_2O, for long term storage in any ordinary freezer (-20°C).
- Prepare a 10X working stock primer mix as per the specified volumes in the table below (can be kept at -20°C).

Primer	**10X concentration (Stock)**	**Volume (µl)**
MG-F3	2 µM	2
MG-B3	2 µM	2
MG-FIP	16 µM	16
MG-BIP	16 µM	16
MG-LB	4 µM	4
ddH_2O	-	60
Total volume (Mix primers)		100

Master Mix

- Identify the number of clinical specimens to be tested, then add a positive control and a negative control to each batch of clinical specimens. This constitutes the number of LAMP tests required.
- **NB:** The LAMP tubes should be labelled and set up accordingly prior to preparing the reaction mix.
- Set up each LAMP reaction by combining the reagents noted in the table below, in the specified quantities. If using Appendix 1 for DNA extraction, add each reagent to a tube in the order mentioned in the table, keeping the LAMP tubes on ice or a cold block during preparation of the reaction.

Reagent	Quantity (µL)
WarmStart Colorimetric LAMP 2X Master Mix	10.0
10X primer mix	2.0
dH_2O	3.0
Extracted DNA	5.0
Total volume per reaction	20.0

Add DNA (Appendix 1) to LAMP tubes

- Flick (to mix gently) the sample and add 2 µL to the appropriate LAMP tube.
- Use positive and negative swabs in your DNA extraction along with test swabs. NB: Positive control DNA extracted from *Mycoplasma gallisepticum* vaccine strain (ts-11) could also be used.
- For the negative control, add 5 µL of DNA extraction buffer.

Run LAMP Test

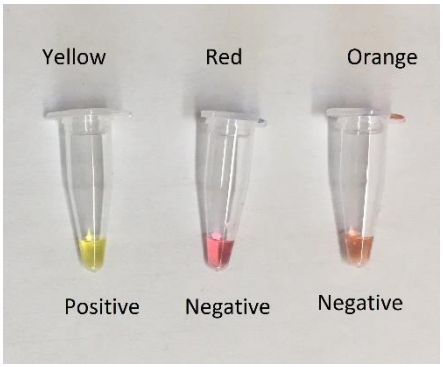
Set up the temperature at 65° C in a heat block.

- Place the LAMP tubes at 65° C for 1 hour.
- Upon completion of the incubation, remove the reaction tubes and interpret the results as below:

  Yellow colour = Positive

  Red colour = Negative

  Orange colour = Negative



The test is invalid if the reaction colour in positive control is red/orange and/or the reaction colour in negative control is yellow.

Appendix 4

LAMP DIAGNOSTIC FOR THE DETECTION OF *Erysipelothrix rhusiopathiae*

PURPOSE

Describes the procedure for performing a colourimetric loop-mediated isothermal amplification (LAMP) assay for the detection of *Erysipelothrix rhusiopathiae* in swabs taken from suspect chickens.

SCOPE

Erysipelas, caused by the bacterium *Erysipelothrix rhusiopathiae* (ER), a gram-positive bacillus that is resistant to environmental factors and impacts a wide range of avian and mammalian hosts, leading to septicaemia, urticarial, or endocardial forms of the disease in birds. The pathogen has been observed in domestic poultry, feral and captive birds, mammals, reptiles, amphibians, and even on fish surfaces. Economically, turkeys are the most affected poultry species, with chickens, ducks, and geese also experiencing severe outbreaks. Swine suffer the most economic impact among mammals, while it can cause polyarthritis in lambs. Turkeys often display cyanotic cutaneous lesions and muscular petechiae. It can also infect humans, causing erysipeloid, an occupational disease marked by localised skin lesions and, rarely, septicaemia (Bobrek et al. 2013). Diagnosis relies on clinical signs, bacterial isolation, and PCR testing. Antibiotics are generally effective in treating acute cases, and vaccination is vital for controlling swine outbreaks. Given its broad host range and emergence in wildlife, vigilance is crucial for disease management and prevention. Molecular diagnostics, specifically PCR and LAMP assays, offer efficient alternatives for detecting ER. The LAMP assay, particularly, can serve as a rapid and field-friendly method suitable for on-farm use without advanced laboratory equipment. This adaptability makes the LAMP assay a valuable diagnostic tool, providing a time-efficient alternative to traditional bacterial culture methods or even PCR for ER detection (Yamazaki et al. 2014).

REFERENCES

- Appendix 5 – DNA extraction method
- Bobrek, K., Gaweł, A., & Mazurkiewicz, M. (2013). Infections with *Erysipelothrix rhusiopathiae* in poultry flocks. World's Poultry Science Journal, 69(4), 803-812. https://doi.org/10.1017/S0043933913000822
- Yamazaki, Y., Oba, E., Kashiwagi, N., Sugita, K., Shiiba, K., Baba, Y., Shimoji, Y., & Yamazaki, W. (2014). Development of a loop-mediated isothermal amplification assay for rapid and simple detection of *Erysipelothrix rhusiopathiae*. Lett Appl Microbiol, 58(4), 362-369. https://doi.org/10.1111/lam.12198

PRIMER DETAILS

Primer	Sequence (5'-3')
ER-F3	ATCGTGGACCAAACAAGC
ER-B3	CTGATTCATGATCCCACATTG
ER-FIP	ACGTCGAACTGTTAGATATGCAAAAGATTTAACACAATTTTCAATCGTCC
ER-BIP	TTGTAATGATGAGCGCGATGAGGGAAAGATTCTCTTGATACTTTGA
ER-LF	CGATTGTCATAATCATCATAGCTC
ER-LB	CTTTTAGATTCTCAAGCCGCGTTA

SAFETY

The appropriate personal protective equipment **(e.g. gloves and laboratory coat)** must be worn when handling samples, LAMP reagents and related products.

MATERIALS

- Autoclaved PCR tubes (0.2 ml) are used for LAMP assay
- Pipette (1-10 µl), calibrated
- Pipette tips (10 µl), autoclave sterilised
- ddH_2O
- WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs, Cat. No. M1800S or M1800L)
- 10X LAMP primer mix (Sigma-Aldrich, custom desalted 50 nmol)
- Crushed ice and esky or cold block
- Heat block to provide 65°C temperature

PROCEDURE

Collection of specimens

- Dry cotton swabs taken from liver, spleen, bone marrow etc. or even isolated bacteria can be used as clinical specimens for field DNA extraction.
- In conjunction with each clinical specimen, a swab known to be positive, and another sterile swab should also be used and processed similarly as positive and negative control, respectively.

DNA extraction protocol

Please see Appendix 5 for instructions.

Primer Mix

- The LAMP assay requires the use of five primers. Lyophilised primer sets can be diluted to 100 µM stock solutions by resuspending in nuclease free ddH_2O, for long term storage in any ordinary freezer (-20°C).
- Prepare a 10X working stock primer mix as per the specified volumes in the table below (can be kept at -20°C).

Primer	10X concentration (Stock)	Volume (µl)
ER-F3	2 µM	2
ER-B3	2 µM	2
ER-FIP	16 µM	16
ER-BIP	16 µM	16
ER-LF	4 µM	4
ER-LB	4 µM	4
ddH_2O	-	56
Total volume (Mix primers)		100

Master Mix

- Identify the number of clinical specimens to be tested, then add a positive control and a negative control to each batch of clinical specimens. This constitutes the number of LAMP tests required.
- **NB**: The LAMP tubes should be labelled and set up accordingly prior to preparing the reaction mix.
- Set up each LAMP reaction by combining the reagents noted in the table below, in the specified quantities. If using Appendix 5 for DNA extraction, add each reagent to a tube in the order mentioned in the table, keeping the LAMP tubes on ice or a cold block during preparation of the reaction.

Reagent	Quantity (µL)
WarmStart Colorimetric LAMP 2X Master Mix	10.0
10X primer mix	2.0
dH2O	6.0
Extracted DNA	2.0
Total volume per reaction	20.0

Add DNA (Appendix 5) to LAMP tubes

- Flick (to mix gently) the sample and add 2 µL to the appropriate LAMP tube.
- Use positive and negative swabs in your DNA extraction along with test swabs. NB: Positive control DNA extracted from one dose of *Erysipelothrix rhusiopathiae* vaccine strain which is applied to a cotton swab could also be used.
- For the negative control, add 2 µL of HotSHOT buffer (a mixture of equal volumes of HotsHOT solution 1 – lysis, and solution 2- neutralising buffer).

Reagent	Quantity (µL)
WarmStart Colorimetric LAMP 2X Master Mix	10.0
10X primer mix	2.0
dH2O	8.0
Extracted DNA-Filter paper	0.0
Total volume per reaction	20.0

Run LAMP Test

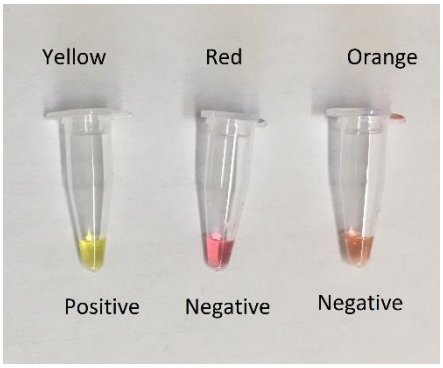
Set up the temperature at 65°C in a heat block.

- Place the LAMP tubes at 65°C for 1 hour.
- Upon completion of the incubation, remove the reaction tubes and interpret the results as below:

  Yellow colour = Positive

  Red colour = Negative

  Orange colour = Negative



The test is invalid if the reaction colour in positive control is red/orange and/or the reaction colour in negative control is yellow.

Appendix 5

DNA EXTRACTION USING HOTSHOT METHOD

PURPOSE

This SOP describes the procedure for field extraction of DNA from clinical swab samples, bacterial cultures and vaccines using the HotSHOT method.

SCOPE

The HotSHOT method for DNA extraction hinges on two essential solutions: the lysis buffer (Solution 1) and the neutralising buffer (Solution 2) (Truett et al. 2000). In Solution 1, sodium hydroxide (NaOH) is the main component responsible for effectively breaking down cell walls and membranes in an alkaline environment. The subsequent addition of Solution 2, containing Tris-Cl, which acts as a neutralising buffer, helps balance and maintain the solution's pH at a level suitable for PCR and LAMP reactions. This method is suitable for DNA extraction from clinical swabs, vaccine samples, and culture specimens. However, it is essential to exercise caution when working with clinical samples that might contain high levels of contaminants.

REFERENCES

- Truett, G. E., Heeger, P., Mynatt, R., Truett, A., Walker, J., & Warman, M. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques, 29(1), 52-54.

SAFETY

The appropriate personal protective equipment **(e.g. gloves and laboratory coat)** must be worn when handling samples, DNA extraction reagents and products.

MATERIALS

- Autoclaved 1.5 ml tubes
- Pipette (200 µl -10000 µl), calibrated
- Pipette tips (200 µl and 1000 µl), autoclave sterilised
- Required reagents:
  - NaOH (Sodium Hydroxide pelleted, Caustic soda) (CSA Scientific, Chem-Supply Australia) Molecular weight: 40.0 gram/L
  - Ethylenediaminetetraacetic Acid Disodium salt dihydrate AR (Di Sodium EDTA) (CSA Scientific, Chem-Supply Australia) Molecular weight: 372.24
  - Tris HCL (Molecular Grade, Thermo Fisher Scientific) Molecular weight: 157.56 gram/L
  - Hydrochloric acid, 1N standard solution, Thermo Scientific Chemicals
- Preparation of HotSHOT solutions
  - Preparation of Solution 1 (lysis buffer)
    : Weigh 0.5 g of NaOH pellets and carefully add them to a clean 500 ml bottle. Use caution when handling NaOH as it is caustic.
    : Weigh 0.04 g of EDTA di sodium salt and add it to the same container.
    : Add 500 mL of sterile dH_2O to the bottle.

: Stir the solution until both NaOH and EDTA di sodium salt are completely dissolved.

: Measure the pH but do not adjust the pH of the Lysis Solution; it should remain around pH > 11.

- Preparation of Solution 2 (Neutralising Solution)

: Weigh 3.15 g of Tris HCl powder and carefully add it to a clean 500 ml bottle.

: Add 500 mL of sterile dH_2O to the bottle.

: Stir the solution until the Tris HCl is completely dissolved.

: Measure the pH of the Neutralising Solution using a pH meter. The pH should be approximately 4.7.

: Adjustment of pH for the Neutralising Solution

- If the pH of the Neutralising Solution is ~ 4.7, slowly and carefully add small amounts of HCl while continuously monitoring the pH with the pH meter. Stir the solution after each addition of HCl and recheck the pH until it reaches around pH 1.9. Be cautious not to overshoot the desired pH.

- Storage: Store the Lysis Solution and Neutralising Solution in separate containers, and label them clearly. Keep the solutions at room temperature and secure their containers to prevent contamination. For optimal results, it is advisable to prepare both solutions fresh or, at a minimum, to prepare them monthly.

PROCEDURE

Collection of specimens and processing

- Use dry cotton swabs collected from gross lesions or isolated bacteria for DNA extraction.
- Prepare a positive swab and one sterile swab to be processed in the same manner as the positive and negative controls, respectively.
- Set up and label one microcentrifuge tube (1.5 ml) for each sample and prepare two additional tubes for the positive and negative controls.
- Add 75 µl HotSHOT solution 1 to each 1.5 ml tube.
- Place the swab sample into the 1.5 ml tube and gently rotate the swab five times to ensure adequate mixing.
- Incubate each tube at 100°C in a hot block for 10 minutes.
- After incubation, remove the tubes from the hot block and add 75 µl of HotSHOT Solution 2 to each tube.
- Remove the swab and extract as much liquid as possible by pressing the swab against the sides of the tube.
- Discard the swab properly.
- Use the solution as the source of DNA template in the subsequent LAMP reaction or PCR.

Waste disposal

All waste material is to be placed in the laboratory biological waste bins, and then autoclave sterilised prior to disposal.