Detection of *Salmonella* spp. in Laboratory Animal Pellet Feed using Real-Time Polymerase Chain Reaction (qPCR) and Loop-Mediated Isothermal Amplification (LAMP)

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ABSTRACT

Salmonella spp. contamination in animal feed is challenging for the commercial feed industry and laboratory animal facilities. Salmonellosis is a term used for Salmonella infection. Salmonellosis is a zoonosis directly transmitted to humans through contaminated feed, food, water, or infected animals. Nevertheless, infection in animals is mainly caused by contaminated feed. Since it provokes a significantly high economic loss due to its high mortality, accurate and rapid Salmonella detection methods are necessary for monitoring the quality of animal feed. The standard method for Salmonella detection in animal feed, which refers to ISO 6579, is based on the culture method, which takes about 5-7 days. Thus, an alternative method is required to give valid results faster. This study aims to develop alternative methods for Salmonella spp. detection in animal feed using Loop isothermal Amplification (LAMP) and Real-time Polymerase Chain Reaction (PCR) methods. Salmonella detection has been carried out on animal feed pellets artificially inoculated with Salmonella Typhimurium *ATCC 14028 at three concentration levels, namely 1, 3, and 9 cfu per test portion (25-gram samples). e-LOD range values between the LAMP method and Real PCR are compared with the culture method and analyzed descriptively. Salmonella detection method using LAMP and Real-Time PCR showed the value e-LOD50 at 0.62 cfu/portion in mouse feed and e-LOD50 at 0.37 cfu/portion in rabbit feed. Compared to the LOD of the culture method, the e-LOD of LAMP and PCR showed similarity. This e-LOD value is the same as the e-LOD culture method. Related laboratories can utilize the results of this study to monitor the quality of animal feed.*

Kontaminasi Salmonella spp. pada pakan hewan merupakan tantangan bagi industri pakan komersial dan juga laboratorium hewan uji. Salmonella dapat menyebabkan penyakit salmonellosis pada hewan uji dengan tingkat mortalitas yang tinggi, sehingga menyebabkan kerugian ekonomi yang signifikan. Penularan penyakit ini kepada manusia dapat terjadi melalui kontak langsung dengan pakan yang terkontaminasi atau melalui hewan terinfeksi yang menularkan Salmonella pada pangan manusia dan sumber air. Pakan hewan merupakan sumber utama penularan Salmonella pada hewan. Karena menyebabkan kerugian yang besar akibat angka mortalitas yang tinggi, metode deteksi Salmonella spp. yang cepat dan akurat diperlukan untuk pemantauan kualitas pakan hewan. Metode deteksi Salmonella yang akurat dan cepat diperlukan dalam pemantauan kualitas pakan hewan. Metode standar untuk deteksi Salmonella pada pakan mengacu pada ISO 6579 yaitu menggunakan metode kultur. Metode kultur membutuhkan waktu pengujian sekitar 5-7 hari, sehingga diperlukan metode alternatif yang memberikan hasil valid dalam waktu lebih singkat. Penelitian ini bertujuan untuk memverifikasi metode alternatif untuk deteksi Salmonella pada pakan menggunakan metode Loop Isothermal Amplification (LAMP) dan Real-time Polymerase Chain Reaction (real-time PCR). Deteksi Salmonella telah dilakukan pada pakan mencit dan kelinci berbentuk pelet yang dicemari dengan Salmonella Typhimurium ATCC 14028 pada tiga tingkat konsentrasi, yaitu ±1, ±3, dan ±9 cfu per porsi uji (25 gram sampel). Nilai rentang e-LOD antara metode LAMP

dan Real-time PCR dibandingkan dengan metode kultur, lalu dianalisis secara deskriptif kualitatif. Metode deteksi Salmonella menggunakan LAMP dan Real-time PCR menunjukan nilai e-LOD$_{50}$ sebesar 0,62 cfu/porsi uji pada pakan mencit dan e-LOD$_{50}$ sebesar 0,37 cfu/porsi uji pada pakan kelinci. Nilai e-LOD ini sama dengan e-LOD metode kultur. Hasil penelitian ini dapat dimanfaatkan oleh laboratorium terkait dalam pemantauan kualitas pakan hewan.

Keywords: *Salmonella*, animal feed, LAMP, PCR
Kata Kunci: *Salmonella*, pakan hewan, LAMP, PCR

1. Introduction

Salmonella spp. is a genus of bacteria from the *Enterobacteriaceae* family, Gram-negative, motile, aerobic or facultatively anaerobic, rod-shaped, negative oxidase, non-spore-forming, measuring about 0.7-1.5 µm wide and 2.0-5.0 µm long (Billah & Rahman, 2024). *Salmonella* spp. is pathogenic to humans and animals (Pal *et al*., 2020). These bacteria grow at 2-54°C, pH 3.7-9.4 (Oludairo *et al*., 2023). *Salmonella* spp. are classified into several subspecies/species of organisms based on O (*somatic*) antigen, phase I H (*flagellar*) antigen, and phase II H antigen (Spickler & Larson, 2005)—another classification system of *Salmonella* spp. is based on the clinical symptoms which are divided into two types, typhoid/typhoidal *salmonella* (TS) with enteric fever symptoms and non-typhoid/non-typhoidal *salmonella* (NTS) (Eng *et al*., 2015). In humans, *Salmonella* spp. infections provoke gastrointestinal infections, systemic infections and enteric fever, while in animals, *Salmonella* spp. Infections cause enteric fever (Billah & Rahman, 2024). Furthermore, all rodents are susceptible to *Salmonella* spp. Infection.

Animal feed is the primary source of *Salmonella* spp. transmission in animals. The presence of *Salmonella* spp. in various feed raw materials, especially those rich in protein, means that feed can act as a direct or indirect route of *Salmonella* spp. Transmission. In pellet feed that has been heated, *Salmonella* spp. can still be detected due to contamination of the production environment or during transportation and storage. *Salmonella* spp. can easily survive in the environment by forming biofilms on the surface of objects, water, and soil (Sargeant *et al*., 2021). *Salmonella enteritidis* can survive for more than 26 months in feed artificially contaminated with *Salmonella* spp. (Meerburg & Kijlstra, 2007). Therefore, routine monitoring of *Salmonella* spp. in animal feed is necessary (Sargeant *et al*., 2021). The presence of *Salmonella* spp. in feed can also affect human health through direct contact with contaminated feed or infected animals to human food and water sources (Sargeant *et al.*, 2021).

Salmonella spp. can be isolated from non-selective pre-enrichment media and cultured on selective media containing inhibitors. Furthermore, Salmonella spp. can be confirmed using bacteriological or molecular methods (Pal *et al*., 2020). Testing with the culture provides the best results for detecting *Salmonella* spp. However, this test generally takes a long time, around 5-11 days. In ISO 6579, the standard method of *Salmonella* spp., the testing procedure is carried out using pre-enrichment media in the form of *buffered peptone water* (BPW), followed by enrichment on modified semisolid Rappaport-Vassiliadis (MSRV) media and isolation on xylose-lysine-deoxycholate (XLD) agar and selected plate media as complementary. This method is characterized by its simplicity and economic efficiency (Demirbilek, 2018) compared to molecular methods such as polymerase chain

reaction (PCR). However, the PCR method offers the advantage of a shorter testing duration even though it comes with the disadvantage that it costs more.

In addition to PCR, another molecular method applicable for detecting *Salmonella* spp. is loop-mediated isothermal amplification (LAMP). This method is used to detect pathogens in clinical specimens and feed matrices. LAMP is a novel nucleic acid amplification test (NAAT) that has become an alternative to PCR testing because it can rapidly detect various bacteria, fungi, parasites and viruses. In 2005, Hara-Kudo et al. conducted a study on detecting *Salmonella* spp. in eggs artificially inoculated with *Salmonella* spp. The study results stated that the LAMP method is fast, specific and sensitive for detecting *Salmonella* spp. (Yang *et al.*, 2021).

The LAMP testing process is faster than the PCR method because it can amplify target DNA more efficiently, with 10^9 in one hour, while PCR generally takes 1-2 hours and produces almost 20 times less DNA than LAMP. Another advantage of the LAMP method is its high tolerance to biological substances in clinical and food samples. In contrast, PCR is generally susceptible to various inhibitors contained in food or feed matrices. Therefore, LAMP is preferable (Yang et al., 2021).

After that, various tests for *Salmonella* spp. in food samples using the LAMP method continued to develop. They began to develop in animal feed samples, but there were no studies on testing *Salmonella* spp. with LAMP and PCR in laboratory animal pellet feed samples (Yang et al., 2018). Various alternative methods have been developed to detect *Salmonella* spp., but they still cannot replace the culture method (Demirbilek, 2018).

Because *Salmonella* spp. is a pathogen harmful to animals and can be transmitted to humans, including through feed, the Federation of European Laboratory Animal Science Associations (FELASA) recommends that Salmonella spp. testing in laboratory animals is carried out routinely every three months.

This study aims to develop an alternative method of *Salmonella* testing in laboratory animal feed that can provide fast and valid results. In this study, verification of *Salmonella* spp. Detection tests in laboratory animal feed using PCR and LAMP as alternative methods were conducted. The animal feed used in this test is mice and rabbit feed in pellets. Mice feed has a different content of water, fiber, and other nutrients, so it becomes a matrix that can affect the survival rate of *Salmonella* spp. Related laboratories can use the results of this study to monitor the quality of laboratory animal feed.

2. Methodology

2.1. Time and Place of Research

This research was conducted at the National Quality Control of Drug and Food (NQCLDF), Indonesia Food and Drug Authority, in October-November 2022.

2.2. Research Materials and Instruments

The materials used in the study were laboratory animal feed for mice and rabbits in the form of pellets and raw microbes *of Salmonella* Typhimurium ATCC 14028 obtained from the Laboratory of Microbiology and Molecular Biology of NQCLDF, Indonesian FDA.

The media used were Buffered peptone water (BPW), Rappaport Vassiliadis-Soya (RVS) Xylose Lysine Deoxycholate (XLD), Brilliant Green Agar (BGA), Tryptic Soy Agar

(TSA), Plate Count Agar (PCA) and Bacto™ Peptone. The reagents used were PCR reagents from iQ-Check *Salmonella* spp. II BIORAD® ready-to-use PCR Reagent kit consisting of Lysis reagent, Fluorescent probes, Amplification mix, PCR negative control and PCR positive control, LAMP reagent from 3M™ Molecular Detection Assay 2 - *Salmonella* consisting of Color Coded Reagent Tubes, Pre-Dispensed and Ready-To-Use Lysis Solution (LS), Extra Caps, Reagent Control Tubes, Quick Start Guide, 70% Alcohol, distilled water/ddH2O, Cleaning reagent (DNA/RNase AWAY), Bleach (NaOCl) 5%, Sodium chloride 0.85% and McFarland standard.

Research Instruments: The instruments used in this study are *Real-time* PCR (qPCR) and Loop-Mediated Isothermal Amplification (LAMP), a computer that already contains real-time PCR software and 3M™ Molecular Detection System software, Stomacher, Vortex, Centrifuge plate rotor, Centrifuge tube 1.5 mL rotor, spin down, heating block, cooling block, analytical balance, hot plate, autoclave deconstruction and autoclave sterilization, Bio Safety Cabinet (BSC), Laminar Air Flow (LAF), Incubator 36±2° C Incubator 41.5±1° C and Vitek 2 compact system instrument.

2.3. Sample Preparation

The test samples were divided into three types, namely sample A (sample without microbial addition), sample B (sample added with standard microbes at several levels of contamination) and sample C (dilution media contaminated with standard microbes at high concentrations). Sample preparation was carried out based on the sample preparation method in ISO 6887-1:2017. Samples consisted of mice and rabbit feed in the form of pellets, weighed as much as 25 grams and then mashed using a sterile mortar and pestle. The feed was put into a sterile screw cap bottle containing 225 mL of BPW solution. In sample A, nothing was added to the sample mixture. In sample B, a bacterial inoculum spike was added from the standard microbe *Salmonella* Typhimurium ATCC 14028 with several dilution levels from 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. In sample C, a high concentration of bacterial inoculum was added to the BPW medium.

2.4 Preliminary Test of Standard Microbial

This preliminary test was conducted to determine the concentration of the microbial standard by calculating the Total Plate Count (TPC) on *Salmonella* Typhimurium ATCC 14028. The microbial standard was mixed into 0.85% NaCl solvent, and the turbidity level was compared with 0.5 Mc Farland, then dilutions were increased to 10^{-8}. The microbial standard from the dilution levels of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} was taken 1 mL and put into a Petri dish and then mixed with PCA media with the pour plate method and then incubated at 37 ± 1 °C for 24 hours for TPC counting.

2.5 Determination of Limit of Detection (LOD)

The alternative method should give the same or better results than the standard method (culture method). The potential contamination of *Salmonella* spp. with low concentrations in animal feed causes the need to determine the LOD value for each *Salmonella* spp.—detection test method used in the laboratory. LOD determination was carried out on samples A and B, referring to ISO 16140-3: 2021, with an *experimental design* using protocol 1 (appendix 1). Bacterial inoculum from the microbial standard with a known concentration in the preliminary test was added to sample B. This inoculum was made into

three levels of contamination. The first is low contamination level (1 x LOD_{50} colonies/test portion with four replications), the second is medium contamination level (3 x LOD_{50} colonies/test portion with four replications), the third is high contamination level (9 x LOD_{50} colonies/test portion with one replication) and testing sample A (sample without microbial addition with one replication).

Since there is no information regarding the LOD_{50} method for this animal feed sample, the LOD $value_{50}$ is considered one colony/test portion. To obtain concentrations of ±1, ±3 and ±9 CFU/test portion, *Salmonella* inoculum containing approximately 1, 3 and 9 colonies was added to the sample mixture according to the TPC results in the preliminary test. To determine the actual bacterial concentration of the *spiking* that has been done, the TPC calculations were made again at dilutions of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} of microbial spiking in sample B. Furthermore, the spike of sample C was carried out by adding standard microbial inoculum into BPW media using a bacterial inoculum with a high concentration according to the ALT results in the preliminary test.

2.6 Test stages

2.6.1 Non-selective Enrichment Stages (Enrichment Sample)

All samples described in point 2.5 were homogenized and incubated at 36 ± 2 °C for 18 ± 2 hours. This stage is a selective pre-enrichment stage on BPW enrichment media by ISO 6579-1: 2017. Samples that have finished incubating for 18 ± 2 hours are then tested in parallel by PCR, LAMP and culture methods.

2.6.2 PCR test stages

DNA Extraction. Extraction stages were performed using the procedures in the PCR reagent kit manual (iQ-Check *Salmonella* spp. II BIORAD®). In samples that have been enriched, gently shaking is done, and as much as 1 mL is taken and then put into a 1.5 mL tube. *Shaking* too much is avoided so that food debris is not carried away. Next, centrifugation of the sample was carried out at a speed of 10,000-12,000 x g for 5 minutes. The supernatant formed was discarded, then 200 µL of lysis reagent was added to the sample sediment, resuspended and vortexed the sample until homogeneous. The sample was placed on a dry heat block with a temperature of 95˚ C - 100˚ C for 10-15 minutes, then a second homogenization using a *vortex* and a second centrifugation with the same speed and time as the first. The supernatant formed after this process was transferred to a 1.5 mL tube for further PCR analysis.

Preparation of PCR mix and addition of Template DNA. The reaction mix was prepared using 40 µl Amplification mix and five µl Fluorescent probes in each well. A total of 45 µl reaction mix was piped into each well, adjusted to the number of samples to be run. A total of 5 µl of DNA extracted from samples A, B, and C, positive control kit and negative control kit were each included in the wells. Centrifugation ensures no bubbles in the wells for 1 minute (quick spin). This centrifugation stage also intends to reduce the reagents still attached to the sound wall. The plate is inserted into the Real-time PCR machine, and the plate placement is ensured by the PCR map that has been made.

Data Analysis and Interpretation of Results. This stage is performed on the appropriate PCR software, and the data is interpreted according to the kit manual's

requirements. Verifying the validity of positive and negative controls was performed before analyzing the sample results. If both controls meet the requirements, the controls and samples are declared valid according to the validity requirements presented in Tables 1 and 2.

Table 1. Validity of positive control and negative control on PCR kit reagents

Parameters	*Salmonella* spp. detection (FAM channel)	Internal Control Detection (HEX channel)
Negative Control	Cq = N/A*	28 ≤ Cq ≤ 40
Positive Control	26 ≤ Cq ≤ 36	N/A

*A Cq value indicating N/A (not applicable) occurs when the sample's fluorescence does not appear significantly above the noise or crosses the threshold.

Table 2. Validity of test samples on PCR kit reagents

Salmonella spp. detection (FAM channel)	Internal Control Detection (HEX channel)	Interpretation
Cq ≥ 10	N/A	Positive
Cq = N/A	Cq ≥ 28	Negative
Cq = N/A	Cq = N/A	Inhibition*

*When the sample and internal control have a Cq value = N/A, the sample should be diluted at 1:10 and retested.

2.6.3 Stages of LAMP Test

DNA Isolation. DNA isolation stages were carried out by the procedures in the LAMP reagent kit manual (3M™ Molecular Detection System and 3M™ Molecular Detection Assay 2). The tube containing Ready-to-Use Lysis Solution (LS), which still has a lid, is flipped before use so that it is homogeneous. The tube cap was then opened using a lysis tube, disappeared, and the cap was removed. A total of 20 µL of the sample mixture with BPW was introduced into the LS tube. One LS tube without a sample was prepared as negative control and another as reagent control. The tubes were then put into a heating block and heated at 100°C for 15 min without capping. The LS liquid, which is pink before heating, will turn yellow when heated. After heating, the tubes are cooled using a heating block at room temperature (20-25°C) for 5 minutes. The colour of LS liquid will return to pink after cooling.

Amplification. Colour-coded reagent Tubes were opened using a reagent tube disappear, and then the caps were removed. A total of 20 µL of DNA isolation results were taken from the top of the LS tube (to avoid taking from the bottom of the tube) and inserted into a tube containing lysis reagent, then homogenized by resuspension. A total of 20 µL each of LS from negative control and LS control reagents were also put into Color-Coded Reagent Tubes. The tubes filled with samples, negative controls and reagent controls were then closed using a new tube cap. The closed tubes were inserted into the loader tray, and the 3M™ Molecular Detection System connected to a PC was run according to the tool manual. The results were read and viewed on the LAMP software.

Interpretation of Results. Results are positive if a peak appears, and a positive symbol is in the image. Results are negative if there is no peak and a negative symbol in the image—salmonella spp. Detection results using 3M™ can be reported if the negative control and reagent control provide valid results.

2.6.4 Stages of the culture method

2.6.4.1. Selective Enrichment Stages for the Culture Method

Pre-enrichment culture from each sample in BPW was introduced into 0.1 mL of *Rappaport Vassiliadis-Soya* (RVS) medium and incubated at 41.5 ± 1°C for 24 ± 3 hours (ISO 6579-1:2017).

2.6.4.2. Isolation on Selective Media Culture Method

One clutch of culture from RVS media was inoculated on the surface of XLD and BGA selective media and then incubated at 36 ± 2°C for 24 ± 3 hours. Colonies that grew on the media were observed. On XLD media, *Salmonella* spp. Positive cultures were characterized by red colonies with or without a black spot in the center. On BGA media, Salmonella spp. Colonies are characterized by transparent, colourless, pink, or cloudy white colonies with a pink-to-red zone around them (ISO 6579-1:2017).

2.6.4.3. Biochemical test confirmation

Specific colonies on BGA and XLD media were then inoculated on the surface of TSA media **and** incubated at 36 ± 2°C for 24 ± 3 hours. The growing colonies were identified using the Vitek 2 *compact system* instrument.

2.7 Data Analysis

Data were measured using descriptive statistics by comparing qualitative data from each bacterial growth medium with the instruments used for confirmation tests.

3. Results and Discussion

3.1. Total Plate Numbers of *Salmonella* Typhimurium ATCC 14028

Stock suspensions of Salmonella spp. were counted using the Total Plate Count (TPC) method to obtain the spike concentration. The TPC results of *Salmonella* spp. 0.5 Mc Farland are as follows:

Table 3. Total Plate Count value of *Salmonella* Typhimurium ATCC 14028

Microbial Test	Dilution							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Simple	TMTC	TMTC	TMTC	TMTC	TMTC	542	63	7
Duplo	TMTC	TMTC	TMTC	TMTC	TMTC	545	44	10
Average	TMTC	TMTC	TMTC	TMTC	TMTC	543,5	53,5	8,5

Description: TMTC = Too many to count

Table 3 shows the proportional growth of *Salmonella* spp. from low to high dilutions. At low dilutions, the number of colonies was too many to count (above 550 colonies/plate).

Colonies can be counted from dilution 10^{-6} until a unit value is obtained at dilution 10^{-8}. The TPC values are calculated from the average number of colonies at dilutions, which shows growth of less than 300 in Petri dishes. In the preliminary test, the total plate count (TPC) of *Salmonella* Typhimurium ATCC 14028 was 53.5 x 10^7 cfu/mL. This TPC value was then used as a reference value in contaminating each feed sample with a concentration of 1xLOD_{50}, 3xLOD_{50}, and 9xLOD_{50} colonies per test portion using 25 grams of sample. The e-LOD_{50} value in each sample will be calculated based on the *real* TPC value of *Salmonella* spp at the time of treatment and testing in each sample.

3.1 Determination of LOD for *Salmonella* Detection Method in Pelleted Mouse Feed

The *Salmonella* spp. Detection methods used in this study were the culture method, a molecular method using Loop-mediated isothermal amplification (LAMP) technique and Real-time Polymerase Chain Reaction (Real-time PCR). Samples prepared and contaminated with *Salmonella* spp. with several concentrations are tested using these three methods. The LOD test results can be seen in Table 4.

Table 4. Detection test results of *Salmonella* spp. in mice pellet feed samples using the culture method

Treatment	Repeatability	*Concentration of contaminants*	Culture Method				Biochemical Confirmation
			BPW	RVS	XLD	BGA	
Sample B 1 x LOD50	1	1 cfu	+	+	+	+	very good identification
	2	1 cfu	+	-	-	-	NA
	3	1 cfu	+	+	+	+	very good identification
	4	1 cfu	+	-	-	-	NA
Sample B 3 x LOD50	1	3 cfu	+	+	+	+	very good identification
	2	3 cfu	+	-	-	-	NA
	3	3 cfu	+	+	+	+	very good identification
	4	3 cfu	+	+	+	+	very good identification
Sample B 9 x LOD50	1	9 cfu	+	+	+	+	very good identification
Sample C	1	9 cfu	+	+	+	+	very good identification
Sample A	1	NA	-				
Media Control	1	NA	-				

*NA = not available

Table 4 shows the concentration level of *Salmonella* spp. In a spike of as much as one cfu/test portion, Salmonella spp. can be detected again in 2 out of 4 replicates, while for a spike of three cfu/test portion, it can be detected again in 3 out of 4 replicates. As for the spike of nine cfu/test portion, it was detected in 1 out of 1 test replicates.

Table 5. Detection test results of *Salmonella* spp. in pelleted mice feed samples using Real-time PCR method

Contaminants	Repeatability (Cq value)			
	1	2	3	4
Sample B (1 x LOD50)	24,44	Undetermined	22,91	Undetermined
Sample B (3 x LOD50)	22,22	Undetermined	20,74	20,7
Sample B (9 x LOD50)	19,65			
Sample C	14,55			
Sample A	Undetermined			
Media Control	Undetermined			

Table 5 shows the detection test results of *Salmonella* spp. in mice feed samples using the *Real-time* PCR method. The cq value of the amplification cycle when the curve cuts the *threshold* limit so it can provide information that the target DNA is detected in the tested sample. The Cq value is inversely proportional to the sample's target DNA amount. The smaller Cq value indicates that the amount of target DNA is increasing so that the target DNA can be detected in the initial PCR cycle—samples contaminated with *Salmonella* spp. A concentration of 9 cfu showed the lowest Cq value compared to samples contaminated with three cfu and one cfu. The table above shows that at the concentration level of *Salmonella spike* one cfu / test portion, *Salmonella* spp. can be detected again in 2 out of 4 replicates. In contrast, for *spike* three cfu / test portion, *Salmonella* spp. can be detected again in 3 out of 4 replicates. As for the *spike* of 9 cfu/test portion, *Salmonella* spp. was detected in 1 out of 1 test replicates. These results are 100% in agreement with the culture method test results.

Table 6 shows the results of the *Salmonella* spp. Detection test on pellet-shaped mice feeds samples using the LAMP method**.** From the results above, the concentration of *spike* one cfu / test portion, *Salmonella* spp., can be detected again in 2 out of 4 replicates. In contrast, for spike three cfu / test portion, Salmonella spp. can be detected again in 3 out of 4 replicates. As for the spike of 9 cfu/test portion, *Salmonella* spp. was detected in 1 out of 1 test replicates. These are 100% with the test results using culture and real-time PCR methods.

Table 6: Detection test results of *Salmonella* spp. in pelleted mice feed samples using LAMP method

Contaminants	Repeatability			
	1	2	3	4
Sample B (1 x LOD50)	+	-	+	-
Sample B (3 x LOD50)	+	-	+	+
Sample B (9 x LOD50)	+			
Sample C	+			
Sample A	-			
Media Control	-			

Table 7. Comparison of detection test results of *Salmonella* spp. in mice pellet feed samples using culture, LAMP and Real-time PCR methods

Treatment	Replication	Concentration of contaminants	Culture Method	PCR	LAMP
Sample B 1 x LOD50	1	1 cfu	+	+	+
	2	1 cfu	-	-	-
	3	1 cfu	+	+	+
	4	1 cfu	-	-	-
Sample B 3 x LOD50	1	3 cfu	+	+	+
	2	3 cfu	-	-	-
	3	3 cfu	+	+	+
	4	3 cfu	+	+	+
Sample B 9 x LOD50	1	9 cfu	+	+	+
Sample C (Control Salmonella)	1	9 cfu	+	+	+
Sample A	1	NA	-	-	-
Media Control	1	NA	-	-	-

Table 7 shows that at all spiking concentrations, the three methods show the same results, so the e-LOD values for the three methods can be determined based on the number of positive results for each level of contamination using Protocol 1 ISO 16140-3: 2021, which can be seen in table 8.

Table 8. e-LOD values for mice feed using PCR, LAMP and Culture Methods

Sample	Contamination level				e-LOD50 cfu/test portion
	High level 9 x LOD50 /test portion	Intermediate level 3 x LOD50 / test portion	Low level 1x LOD50 /test portion	Blank	
Mouse Feed	1/1	3/4	2/4	0/1	1.3* x LIL** 1,3* x 0,48
	Real inoculum (cfu) at each level				
	4,2	1,4	0,48		**e-LOD$_{50}$ = 0.62**

* Value is determined based on the e-LOD determination table$_{50}$ ISO 16140-3: 2021 (attachment 1)
** LIL=Low Inoculum Level. The LIL value is determined based on the TPC test result of 40 x 10^7 cfu/mL, considering the pipetting volume and dilution factor of the suspension taken

Protocol 1 of ISO 16140-3:2021 was used to determine e-LOD$_{50}$ because, in this study, the concentration of inoculum in the test portion is uncertain. The preliminary test to calculate the suspension of the test inoculum is used as a reference for calculating the volume of suspension to be included in the sample. The actual calculation of the inoculum concentration must be determined by calculating the TPC when testing the sample, so there may be a shift in the TPC value.

Table 9. The TPC value of *Salmonella* Typhimurium ATCC 14028 stock suspension used as *spike* for mice feed samples

Microbial Test	Dilution							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Simplo	TMTC	TMTC	TMTC	TMTC	TMTC	361	48	6
Duplo	TMTC	TMTC	TMTC	TMTC	TMTC	367	32	12
Average	TMTC	TMTC	TMTC	TMTC	TMTC	364	40	9

Description: TMTC = Too many to count

The total plate count (TPC) test result of *Salmonella* Typhimurium ATCC 14028 stock suspension used as a spike for pellet-shaped mouse feed samples (Table 9) is 40 x 10^7 cfu/mL. This TPC value determines the low inoculum level (LIL) used during the study. The LIL value is then used to determine the e-LOD value$_{50}$ method on mouse feed samples.

The e-LOD value$_{50}$ for culture, LAMP and PCR methods on mice feed showed the exact value of 0.62 cfu/test portion. This e-LOD value of 50 is the estimated LOD value for the entire procedure, including the enrichment stage. The acceptability requirement of e-LOD$_{50}$ < 4.0 cfu/test portion, so these three methods meet the requirements (ISO 16140).

3.2 Determination of LOD for *Salmonella* Detection Method in Pelleted Rabbit Feed

Pelleted rabbit feed samples contaminated with *Salmonella* spp. have also been tested using culture methods, molecular methods using LAMP and Real-time PCR with the following results:

Table 10. Detection test results of *Salmonella* spp. in pelleted rabbit feed samples using the culture method

Treatment	Replication	Concentration of contaminants	Culture Method				Biochemical Confirmation
			BPW	RVS	XLD	BGA	Biochemical identification
Sample B 1 x LOD50	1	1 CFU	+	-	-	-	NA
	2	1 CFU	+	+	+	+	very good identification
	3	1 CFU	+	+	+	+	NA
	4	1 CFU	+	+	+	+	very good identification
Sample B 3 x LOD50	1	3 CFU	+	+	+	+	NA
	2	3 CFU	+	+	+	+	very good identification
	3	3 CFU	+	+	+	+	very goodt identification
	4	3 CFU	+	+	+	+	very good identification
Sample B 9 x LOD50	1	9 cfu	+	+	+	+	very good identification
Sample C (control *Salmonella*)	1	9 cfu	+	+	+	+	
Sample A	1	NA	-				
Media Control	1	NA	-				

In **Table 10**, the concentration level of Salmonella spp. during the spike one cfu /test portion, Salmonella spp. can be detected again in 3 out of 4 replicates, while during the spike three cfu/test portion, it can be detected again in all replicates. As for the spike nine cfu/test portion, *Salmonella* spp. was detected in 1 out of 1 test replicates.

Table 11 shows the detection test results of *Salmonella* spp. in rabbit feed samples using the *Real-time* PCR method. The Cq value is inversely proportional to the sample's target DNA amount. The smaller Cq value indicates that the amount of target DNA is increasing so that

the target DNA can be detected in the initial cycle of PCR—samples contaminated with *Salmonella* spp. A concentration of 9 cfu showed the lowest Cq value compared to samples contaminated with three cfu and one cfu at the concentration level of *Salmonella* spp. In spike one cfu/test portion, *Salmonella* spp. can be detected again in 3 out of 4 replicates, while for spike three cfu/test portion, *Salmonella* spp. can be detected again in all replicates. As for the spike of 9 cfu/test portion, *Salmonella* spp. was detected in 1 out of 1 test replicates. This result is 100% with the test results using the culture method.

Table 11. Detection test results of *Salmonella* spp. in rabbit pellet feed samples using Real-time PCR method.

Contaminants	Replication (Cq value)			
	1	2	3	4
Sample B (1 x LOD50)	Undetermined	15,49	14,96	15,01
Sample B (3 x LOD50)	15,79	17,01	18,46	15,38
Sample B (9 x LOD50)	13,93			
Sample C	14,18			
Sample A	Undetermined			
Media Control	Undetermined			

Table 12. Detection test results of *Salmonella* spp. in pelleted rabbit feed samples using LAMP method

Contaminants	Replication			
	1	2	3	4
Sample B (1 x LOD50)	-	+	+	+
Sample B (3 x LOD50)	+	+	+	+
Sample B (9 x LOD50)	+			
Sample C	+			
Sample A	-			
Media Control	-			

Table 12 shows the detection test data of *Salmonella* spp. in rabbit pellet feed samples using the LAMP method. At the *Salmonella* spp. *spike* concentration level of 1 cfu/test portion, *Salmonella* spp. It could be detected in 3 out of 4 replicates, while for spike three cfu/test portion, *Salmonella* spp. it could be detected in all replicates. As for the *spike* nine cfu/test

portion, *Salmonella* spp. was detected in 1 out of 1 test replicates. This result is 100%, and the test results were obtained using culture and real-time PCR methods.

Table 13. Comparison of detection test results of *Salmonella* spp. in pelleted rabbit feed samples using culture, LAMP and Real-time PCR methods

Treatment	Replication	Concentration of contaminants	Culture Method	PCR	LAMP
Sample B 1 x LOD50	1	1 cfu	-	-	-
	2	1 cfu	+	+	+
	3	1 cfu	+	+	+
	4	1 cfu	+	+	+
Sample B 3 x LOD50	1	3 cfu	+	+	+
	2	3 cfu	+	+	+
	3	3 cfu	+	+	+
	4	3 cfu	+	+	+
Sample B 9 x LOD50	1	9 cfu	+	+	+
Sample C	1	9 cfu	+	+	+
Sample A	1	NA	-	-	-
Media Control	1	NA	-	-	-

*NA = *not available*

Table 14. e-LOD values for rabbit feed using PCR and LAMP

Sample	Contamination level				e-LOD50 cfu *portion*
	High level 9 x LOD50 /test portion	Intermediate level 3 x LOD50 / test portion	Low level 1 x LOD50 /test portion	*Blank*	
Rabbit Feed	1/1	4/4	3/4	0/1	0.5* x LIL 0,5 x 0,74
	***Real* inoculum (cfu) at each level**				
	6,51	2,17	0,74		**e-LOD50 =**

*Values obtained from ISO 16140-3:2021 Table (appendix 1)
**LIL=Low Inoculum Level. The LIL value was determined based on the TPC test result of 62 x 10^7 cfu/mL, considering the pipetting volume and dilution factor of the suspension taken.

Table 13 shows that at all *spiking* concentrations, the three methods show the same results, so the e-LOD values for the three methods can be determined based on the number of positive results for each level of contamination using Protocol 1 ISO 16140-3: 2021.

Table 15. The TPC value of *Salmonella* Typhimurium ATCC 14028 stock suspension used as a spike for rabbit feed samples

Microbial Test	Dilution							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Simplo	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	60	8
Duplo	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	65	5
Average	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC	62,5	6,5

Description: TMTC = Too many to count

The total plate count (TPC) test result of *Salmonella* Typhimurium ATCC 14028 stock suspension (Table 15) was used as a spike for rabbit feed samples, and it was 62.5 x 10^7 cfu/mL. This TPC value is used to determine the low inoculum level (LIL), which is the low inoculum level used during the study; then, the LIL value is used in determining the e-LOD value$_{50}$ method on rabbit feed samples. The e-LOD50 value for culture, LAMP and PCR methods on rabbit feed showed the exact value of 0.37 cfu/test portion. This e-LOD value of 50 is the estimated LOD value for the entire procedure, including the enrichment stage. The acceptability requirement of e-LOD$_{50}$ < 4.0 cfu / test portion so that these three methods meet the acceptability criteria for method verification (ISO 16140). This study conducted secondary validation (verification) for the LAMP and Real-time PCR methods using previously validated reagents/kits. The LAMP method for *Salmonella* spp. has been validated against AOAC Official Method 2016.01 and by AFNOR Certificate No. 3M 01/16-11/16 against ISO 6579. *The Real-time* PCR method using the IQ check Salmonella II kit has been approved by AOAC in 2021 for several matrices, including dry dog food, raw ground chicken, and ham, based on the results of the probability of detection (POD) statistical model that there is no difference between the PCR method using iQ check and the standard method. Thus, the LAMP and PCR methods verified in this study can be used as alternative methods for *Salmonella* detection in rabbit feed because they have been proven to perform equivalent to the golden method (culture method).

3.3. Characteristics of culture methods compared to molecular methods

The culture method is the standard for detecting *Salmonella* spp. in food and feed. It is based on the characteristics of microbial growth on BPW non-selective enrichment media, selective enrichment media, and colony growth on selective media, followed by biochemical and serological confirmation. In this study, biochemical confirmation was carried out using a rapid identification instrument with a kit for *Salmonella* spp., while serological test was not carried out as it did not identify the species or strain level.

In this study, molecular methods such as LAMP and real-time PCR applied to feed samples enriched on BPW media showed performance comparable to that of the culture method in mice and rabbit feed. Rapid screening of *Salmonella* spp. in feed, floor dust and *swabs* using molecular PCR techniques requires 2-3 days of testing time, as Ahaduzzaman et al. (2021) reported. Domesle et al. (2021) also reported the LAMP method as a rapid and *robust* method for routine screening of *Salmonella* spp. in raw pet food with an rLOD value of 1 CFU. LAMP showed 100% concordance with BAM culture and Real-time PCR methods. However, there is no research on testing *Salmonella* spp. by LAMP and PCR on laboratory animal pellet feed samples. In this study, LAMP and PCR methods showed the same positive result rate as the culture method according to ISO 6579. The LAMP and PCR confirmed negative samples within 24 hours, while the ISO method took 5 days. The LAMP and Real-time PCR methods also gave faster presumptive positive results than the culture method on mice and rabbit feed samples contaminated with *Salmonella* spp. This indicates that these methods are fast and valid for detecting *Salmonella* spp. contamination in feed.

The LAMP method used in this study uses the principle of *bioluminescence* as a detector. Samples were enriched in BPW non-selective medium for 18 hours, and then DNA in the samples was isolated. The target DNA was amplified using several pairs of primers (including *loop* primers) at a fixed temperature; the amplification results were detected by bioluminescence and read by the LAMP instrument. Presumptive positive results are reported in real-time, while negative results are displayed after the test completion (AOAC, 2019). AOAC also states that this method has been validated for detecting *Salmonella* spp. in dry dog food with an 18-24 hours enrichment time.

The real-time PCR method used in this study is the iQ-Check Salmonella II kit, which can be validated on samples enriched for 21 hours using primers and molecular beacon probes that target specific sequences of the Salmonella genome. DNA isolation was performed on enriched samples on BPW non-selective media. This method has also been validated for Salmonella spp detection testing in dry dog feed and wet cat feed (AOAC, 2021).

Screening of *Salmonella* spp. using LAMP and *Real-time* PCR methods can be completed in about 24 hours, thus providing faster information regarding the microbiological quality of feed to be consumed by laboratory test animals compared to the culture method, which takes about 5-7 days. Testing using PCR and LAMP with the reagent kit used in this study costs about IDR 200,000 to IDR 250,000 per sample, excluding investment for the purchase of instruments and equipment, while the estimated cost of media/reagents for the culture method is about IDR 170,000 per sample. Budget requirements for molecular methods are relatively more expensive than culture methods, but they are faster in obtaining test results.

3.4. *Positive rate of Salmonella* in *spiked* mice and rabbit feed samples

In this study, the *growth* rate of *Salmonella* spp. in rabbit feed was more significant than its growth in mice feed. *Salmonella* spp is detected in 8 out of 9 samples of rabbit feed contaminated with *Salmonella* spp at specific concentration levels. As for the mice feed, *Salmonella* spp. was detected in 6 out of 9 samples of rabbit feed contaminated with *Salmonella* spp at specific concentration levels. Differences in feed composition may affect the growth of *Salmonella* spp. Pelleted feed for mice and rabbits is a complete feed with

vitamins. This study used mice feed with the nutritional content listed on the mice feed label as maximum moisture content of 12%, minimum protein of 20%, max fat of 4%, maximum crude fibre of 4%, calcium of 12% and phosphorus of 0.7%. The nutritional content on the rabbit feed label is in the form of maximum moisture content of 12%, minimum crude protein of 15%, minimum crude fat of 2%, minimum crude fiber of 14%, maximum ash of 14%, calcium of 0.80%, phosphorus at least 0.50%, amino acid lysine at least 0.70%, amino acid methionine + cysteine at least 0.5%, urea ND and harmful for aflatoxin. It appears that the rabbit feed contains amino acids that are not contained in the mice feed and may affect the growth of Salmonella spp. However, further research needs to be done regarding the effect of these amino acids on the growth of *Salmonella* spp.

4. Conclusion

In this study, *Salmonella* spp. detection was carried out using the LAMP method, PCR and culture method. LAMP and Real-Time PCR methods can be used for *Salmonella* spp. detection in commercial rabbit and mice feed with the same e-LOD value as the culture method. In mice feed samples, the e-LOD value$_{50}$ was 0.62 cfu/test portion, while in rabbit feed samples, the e-LOD$_{50}$ was 0.37 cfu/test portion. The LAMP and Real-Time PCR methods can be applied as alternative methods in *Salmonella* spp. detection testing for animal feed that provides valid results in a faster time than the culture method.

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