

## Identification of *Avian Influenza* with the Real-Time Polymerase Chain Reaction (RT-PCR) Method

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**Abstrak**— *Avian influenza* (AI) adalah penyakit *zoonosis* yang ditularkan dari unggas ke manusia. Prevalensi penyakit AI di Indonesia kemungkinan akan menjadi ancaman kesehatan global karena kemudahan mutasi pada setiap generasi, yang membuat patogenitas semakin tinggi. Namun, upaya deteksi masih menggunakan metode konvensional, sehingga diperlukan pencarian deteksi yang lebih canggih. Penelitian ini bertujuan untuk mengidentifikasi proses amplifikasi DNA dan hasil deteksi virus *avian influenza* menggunakan *real-time* PCR. Penelitian kualitatif menggunakan sampel dari BBVet Denpasar pada bulan Mei 2022. Sampel dianalisis menggunakan metode *real-time polymerase chain reaction* (RT-PCR), dan hasil yang diperoleh dianalisis secara deskriptif. Hasil penelitian menunjukkan bahwa metode RT-PCR sangat efektif untuk mendeteksi keberadaan AI secara kuantitatif dan molekuler. Grafik yang melewati ambang batas pada siklus yang lebih rendah dari 40 menunjukkan bahwa sampel positif akan menunjukkan Ct nominal. Tidak ada Ct nominal yang terdeteksi dalam penelitian ini, dan grafik tidak melewati ambang batas, sehingga model diuji negatif untuk virus AI. Proses amplifikasi DNA virus *avian influenza* diawali dengan proses amplifikasi sebanyak 40 siklus, dengan masing-masing tahapan siklus berupa denaturasi pada suhu 94 °C selama 30 detik, *annealing* pada suhu 64 °C selama 60 detik, dan sintesis DNA pada suhu 76 °C selama 30 detik. Satu siklus dinyatakan jika proses amplifikasi telah melewati tiga tahap ini.

**Kata Kunci:** *Avian influenza*, Deteksi virus, *Real-time* PCR, Penyakit Zoonosis

**Abstract**— *Avian influenza* is a zoonotic disease transmitted from poultry to humans. The prevalence of AI disease in Indonesia is likely to be a global health threat because of the ease of mutation in each generation, which makes pathogenicity higher. However, detection efforts still use conventional methods, so a more sophisticated detection search is needed. This research aims to identify the DNA amplification process and the results of detecting *avian influenza* virus using *real-time* PCR. Qualitative research using samples from BBVet Denpasar in May 2022. The samples were analyzed using the real-time polymerase chain reaction (RT-PCR) method, and the results obtained were analyzed descriptively. The results show that the RT-PCR method is very effective for detecting the presence of AI quantitatively and molecularly. A graph that crosses the threshold on cycles lower than 40 indicates that a positive sample will show nominal Ct. No nominal Ct was detected in this study, and the graph did not cross the threshold, so the model tested negative for the AI virus. The DNA amplification process of the *avian influenza* virus begins with an amplification process of 40 cycles, with each cycle stage in the form of denaturation at 94 °C for 30 seconds, *annealing* at 64 °C for 60 seconds, and DNA synthesis at 76 °C for 30 seconds. One cycle is declared if the amplification process has passed these three stages.

**Keywords:** *Avian influenza*, Virus Detection, Real-Time PCR, Zoonotic disease.

### 1. INTRODUCTION

*Avian influenza* (AI), often known as "bird flu," is a disease caused by viruses from the family Orthomyxoviridae (Wang et al., 2017; WHO, 2008). This disease can infect various species of poultry and can be transmitted to humans (*zoonoses*) (Agustiawan et al., 2022). The first case of the bird flu virus infecting humans was reported in Hong Kong in 1997 (Mounts et al., 1999). The AIV subtype H5N1 outbreak reported in the Asian region is categorized as highly pathogenic avian influenza (HPAI) (Damayanti, Wiyono, Nuradji, & Cahyono, 2017; WHO, 2006; WOA, 2022). AI is an RNA virus with a fast transmission ability and a pathogenicity strong enough to infect. The nature of its rapid spread is due to RNA viruses, which quickly mutate and adapt to new environments (Agustiawan et al., 2022; Kencana, Suartha, Kardena, & Nurhandayani, 2020). AI viruses are resistant to environments with a certain period and temperature. Transmission occurs through direct contact and indirect contact (Adnyana, 2022; Martinez, Kencana, & Dibia, 2021; Rehman et al., 2023; Thaha, Rauf, & Bagenda, 2018).

In addition to impacting animal health, avian influenza virus also adversely affects human health. The World Health Organization report shows that AI disease has resulted in the mortality of 163 people in Indonesia (Adisasmito et al., 2013; Daniels, Wiyono, Sawitri, Poermadaja, & Sims, 2012; Putri, Widyaningrum, Sugiyono, & Asmara, 2020; Rehman et al., 2023; WHO, 2008). This occurs due to transmission through poultry to humans and meat consumption, which triggers the entry of the virus into the body and results in mortality and morbidity (Daniels et al., 2012). Damayanti et al. (2017) research shows that the influenza virus (H5N1) is histologically capable of causing severe inflammatory reactions, including nonsuppurative encephalitis, tracheitis, myocarditis, interstitial pneumonia, hepatitis, proventriculitis, enteritis, pancreatitis, nephritis, and bursitis. The results of immunohistochemical tests obtained on native chickens in Indonesia indicate that they are susceptible to virus H5N1 HPAI clade 2.3.2 and have reasonably fast transmission. Furthermore, Hewajuli et al. (2018) stated that the AI virus still threatens poultry farming in Indonesia.

Virus prevention efforts are significant to prevent the emergence of pandemics and endemic diseases in an area. In principle, the diagnosis of avian influenza virus is carried out by serological test methods, isolation, and virus identification (Hewajuli & Dharmayanti, 2014; Hewajuli et al., 2018; Kencana, Suartha, Kardena, & Agustina, 2021). One method to detect AI viruses effectively is the real-time polymerase chain reaction method (Handoyo & Rudiretna, 2000; Karo-karo et al., 2022). The development of polymerase chain reaction (PCR) produces real-time PCR techniques that can be evaluated and quantified directly by computers and software. The working principle of real-time PCR is to

detect and quantify DNA with fluorescence signals. The fluorescence signal will increase as the amplification of DNA increases, allowing results to be obtained when the reaction takes place in the software (Feranisa, 2016).

The Denpasar Veterinary Center (BBVet) is one of the Technical Implementation Units (UPT), which has the main task of investigating and diagnosing animal diseases. BVBet Denpasar serves to detect animal diseases with the real-time PCR method. One of the common diseases found and tested at the BBVet Denpasar biotechnology laboratory is avian influenza. The sample tested is divided into two groups based on the source, namely, active and passive samples (Kencana et al., 2021). Active samples come from the results of BBVet Denpasar's internal surveillance, which is given an A code on the delivery number; passive samples come from customers who send samples to detect the presence of viruses and are given a P code on the sample delivery number. Previous studies have attempted to identify the presence of avian influenza virus antigens in native chickens in the Beringkit Animal Market and Galiran Public Market, Bali, actively resulting in positive smears in the trachea of 5-7% (Musdalifa, Kencana, & Suartha, 2020), but many studies have not identified specific methods to directly assess the presence of AI virus in poultry actively or passively. Until now, information about sample testing techniques to detect avian influenza viruses has been relatively scarce, so better identification methods are needed to identify samples. Avian influenza is often confused with Newcastle disease (ND), infectious laryngotracheitis (ILT), infectious bronchitis (IB), fowl cholera, and Escherichia coli infection (Wibawa et al., 2018). To minimize bias when carrying out laboratory tests, this study aims to explain and identify the process of DNA amplification and the results of detecting avian influenza virus using real-time PCR. Hopefully, this research can be used as a responsive method for accelerating the identification of AI viruses in the laboratory.

## 2. RESEARCH METHODOLOGY

### 2.1 Design and Time of Research

This type of qualitative research was conducted in March 2022 at the Biotechnology Laboratory of the Denpasar Veterinary Center. This study seeks to identify the DNA amplification process and the results of detecting the avian influenza virus using real-time PCR. The analyzed blood samples were sent from poultry farms in Denpasar City to ensure the safety and health of poultry. The overall process follows the procedures of BBVet Denpasar. Data are analyzed descriptively, presented in tables, figures, and narratives, and supported by the literature following the topic of study.

### 2.2. Research Implementation

Each stage of testing is carried out in a separate room. Work is carried out in Biosafety Cabinet (BSC) Level 2 at the RNA extraction stage and laminar flow at the master mix and templating stages. The entire process must be done sterilely so that lab coats, nonpowder gloves, and medical masks are used, which will then be discarded when all stages of testing are completed. The Invitrogen PureLink™ Pro Genomic DNA Purification Kit (Invitrogen™) was used. The steps include.

#### 2.2.1 Master Mix

Tools and materials were prepared and sterilized with 70% alcohol and then placed into the laminar flow. The tools prepared are micropipettes of 10 µl and 100 µl, barrier tips of 10 µl and 100 µl, vortex, spin, and microtube. The materials used are reagents, namely, 2x reaction mix, reverse transcriptase enzymes, premixes (a mixture of primer and probe) for AI viruses, and nuclease-free water. The first step was to take 1 µl of reverse transcriptase enzyme for each sample and put it into a microtube coded according to the sample. Then, each sample was put into a premix of 3 µl, nuclease-free water of 3.5 µl, and a 2x reaction mix of 12.5 µl so that the total volume of the master mix for each sample was 20 µl. Microtubes containing master mix reagents are homogenized by divorcing and spinning for a few seconds. The master mix is then stored in the freezer for the templating process.

#### 2.2.2 RNA extraction

Tools and materials were prepared and sterilized first with 70% alcohol and then fed to BSC level 2. The tools prepared are micropipettes of 200 µl and 1000 µl, barrier tips of 200 µl and 1000 µl, tweezers, permanent markers, microtubes, recovery tubes, spin columns, vortexes, incubators, and microcentrifuges. The materials used are samples and reagents used. The reagents used are lysis buffer, carrier RNA, proteinase K, ethanol absolute, washing buffer, and RNase-free water. Reagents do not need to be sterilized first because spraying 70% alcohol will affect the solution of the reagent. The first step when extracting RNA is to mix lysis buffer and carrier RNA, mixing each lysis buffer as much as 210 µl using a single-channel micropipette and carrier RNA as much as 5.88 µl then put into a microtube, then pipetting so that both reagents are evenly mixed. Then, 25 µl of proteinase K was added, and the sample was coded with a marker on the microtube and homogenized by moving the microtube to form figure eight. Then, a 200 µl sample was added, vortexed, and incubated at 56 °C for 15 minutes.

The next step was to add 250 µl of absolute ethanol, vortex and incubate at room temperature for five minutes; the sample was then centrifuged at 8000 rpm for one minute. Next, as much as 675 µl of liquid was poured into the spin column coded according to the sample and then centrifuged at 8000 rpm for one minute. The collection tube was replaced, and 500 µl of washing buffer was added and centrifuged again at a speed of 8000 rpm for one minute. The liquid was removed, and the addition of the washing buffer was repeated and centrifuged at the same speed and time. After that, the

collection tube was replaced and centrifuged at 14000 rpm for one minute. In the last step, the cells were transferred to a recovery tube, and 50  $\mu$ l of RNase-free water was added. The tube was incubated at room temperature for one minute and then centrifuged at 14000 rpm for one minute. RNA is ready to enter the templating stage. In addition to samples, RNA extraction also used positive and negative controls. A positive control was obtained from positive isolates in the virology laboratory at BBVet Denpasar, which was then diluted into  $10^{-3}$ . The negative control used RNase-free water instead of the sample. The accuracy of the implementation of the method can be seen from the control results. Test results are acceptable if the virus does not detect a negative control, while a virus with a good CT detects a positive control.

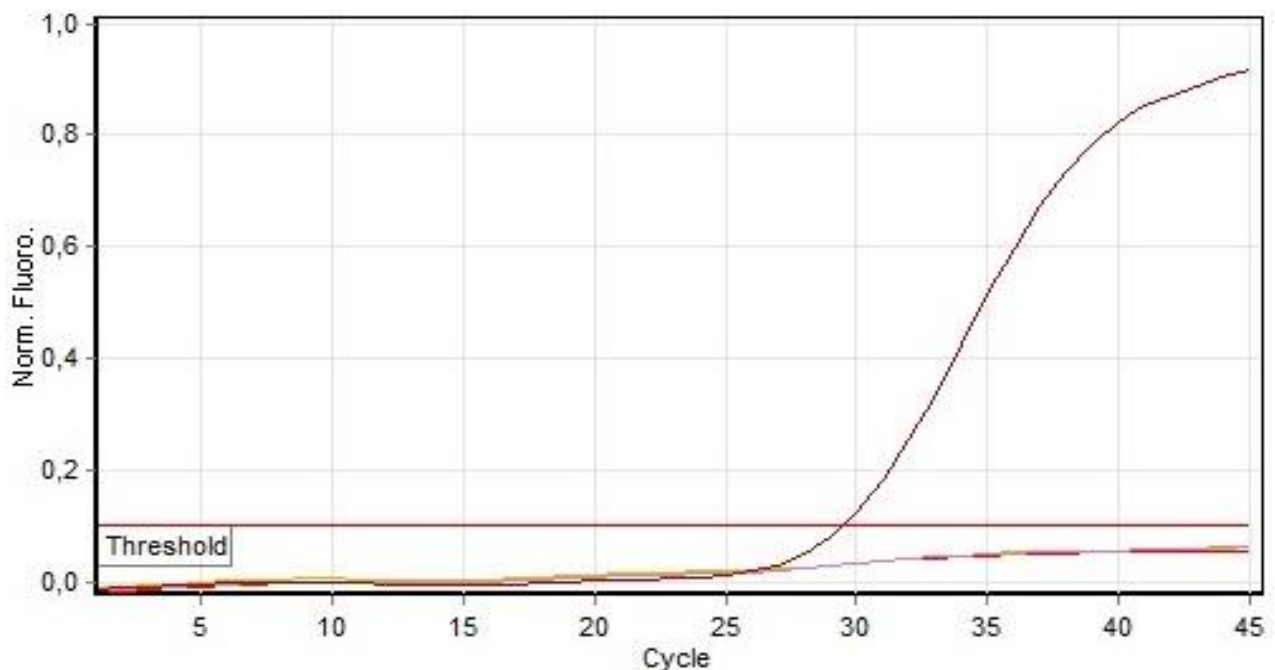
### 2.2.3 Once the RNA and master mix is ready

The next stage is templating. This stage is the mixing of RNA samples with master mixes. The master mix prepared on the microtube was then added to 5  $\mu$ l samples. The total volume of the entire volume on the microtube was 25  $\mu$ l. The microtube was then fed into a real-time PCR machine for amplification. The amplification process until the results are obtained takes approximately one hour and 40 minutes (Musdalifa et al., 2020).

## 3. RESULTS AND DISCUSSION

### 3.1 Detection of avian influenza virus

DNA amplification was performed using machines and real-time PCR software from Qiagen to obtain the amplification results. The results of DNA amplification in this study can be seen in Figure 1. A threshold is a threshold value as a determinant of the presence or absence of viruses in the sample. The criteria for a positive sample are shown by a graph that crosses the threshold in cycles below 40 (Haryanto et al., 2010; Wahyutomo, Ciptaningtyas, & Hadi, 2011).



**Figure 1.** The curve of the AI virus detection graph on sample DNA. Description: *Threshold*, positive or negative threshold line of a sample; *Cycle*, number of cycles; Nominal fluorescence, amount of *fluorescence* signal emitted.

One of the advantages of real-time PCR is that quantitative results are obtained directly from the probe (marker) on the PCR machine. Therefore, there is no need for an electrophoresis process (Feranisa, 2016). Table 1 shows that no AI virus was detected in the tested samples. This is evidenced by the undetected Ct (*cycle threshold*). Ct is quantitative data in the form of nominal cycles when the amount of amplified DNA reaches the threshold, so no detection of Ct means no DNA is amplified. The lower the nominal Ct, the stronger the potential for infection from the virus (Domeracki et al., 2020). Positive and negative controls were used in RNA extraction, and NTC (no template control) was carried out in the master mix process. Each of the controls is used to establish the accuracy of the execution of the method. The results obtained a positive control with a Ct value of 29.51, meaning the control was positive for the AI virus. The negative control does not show a Ct value, which means that the control is harmful to the AI virus. These results show that the accuracy of the results can be trusted. If there is an error, such as a CT number above 40 (false positive), it can be indicated as a reasonable control. This is supported by Adnyana (2021), who states that a variable is used as a control to ascertain whether it is true that a particular independent variable has an influence on another independent variable or if there are other influences.

The H5N1 primer clade 2.3.2.1-specific real-time PCR primer/probe kit was designed and purchased from the CSIRO Australian Animal Health Laboratory. The kit contained one tube each of dry forward primer, dry reverse primer, and dry probe. The forward primer obtained in the kit has 26 nucleotide base sequences with the DNA sequence GCTCCAGAATATATGCATACAAAATTGT. The reverse primer had a 24-nucleotide sequence with the DNA sequence CTATTGGAGTCTGACACCTGGTGT. From these data, it can be determined that the melting temperature of the primary is 72 °C, while the annealing temperature used is 64 °C. The primers mentioned meet the requirements of good primary design indicators so that the accuracy of the amplification process can be trusted. The results of virus quantity on DNA samples are presented in Table 1.

**Table 1.** Results of virus quantity on DNA samples

No	Sample Code	Type	Ct
1	P06210251	Sample	-
2	Neg	Negative Control	-
3	Post	Positive Control	29,51
4	NTC	Master mix control	-

## DNA Amplification Process with Real-time PCR

The process of DNA amplification with real-time PCR is presented in Figure 2. The DNA amplification begins with converting RNA into cDNA using reverse transcriptase enzymes at a temperature of 42–55 °C. The amplification process occurs in several stages, namely, denaturation, annealing, and DNA synthesis. Starting with the denaturation stage, a heating process at a temperature of 94 °C separates the two strands from the DNA molecule. This process needs to be performed at the right time because if the time is too short, the separation of DNA strands may not take place smoothly, while if the time is too long, it will affect enzymes that cannot be exposed to high temperatures (Kurniawati, Sumaryam, & Hayati, 2019).

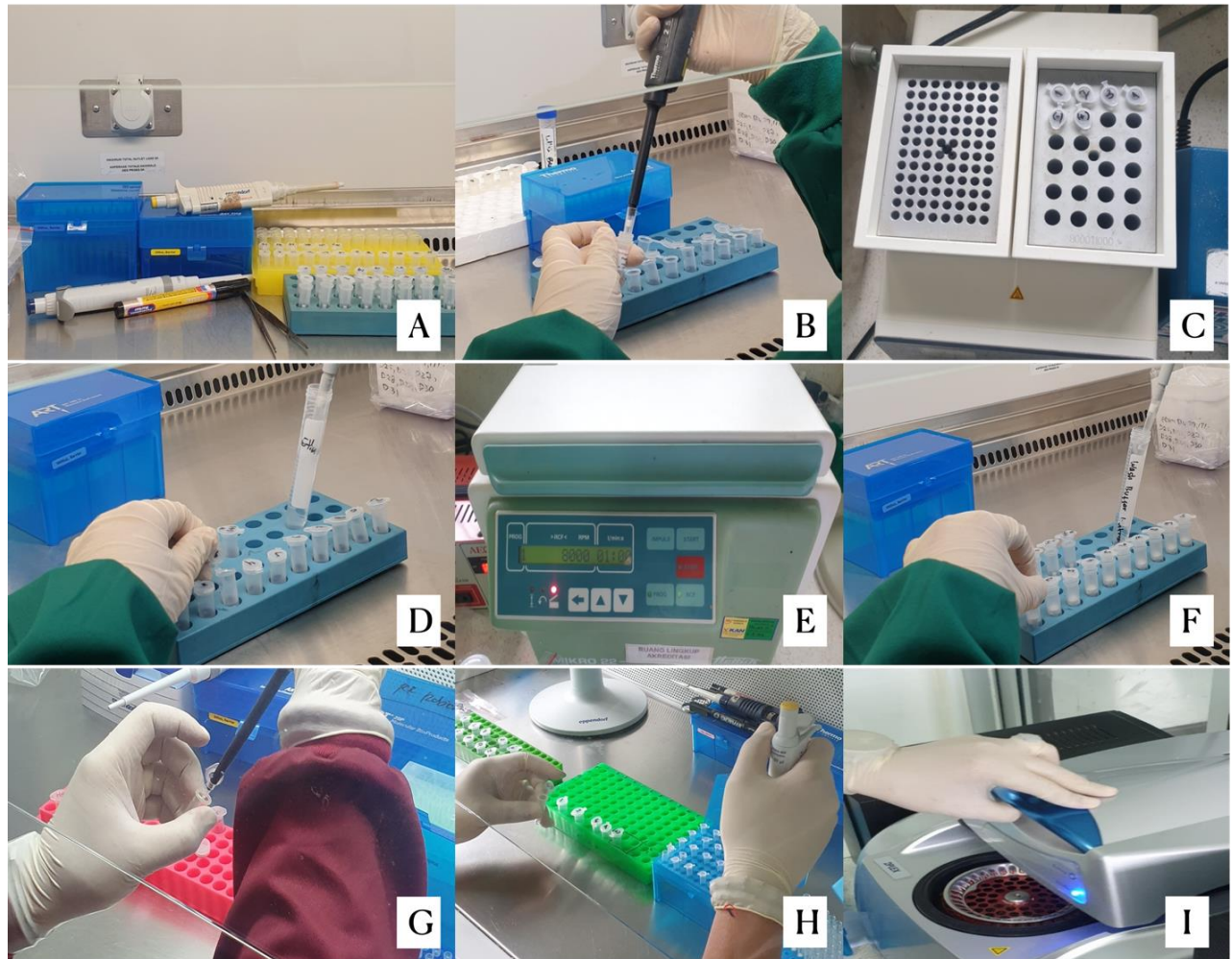
The next stage is annealing, which is the stage of lowering the temperature to 64 °C so that the temperature is in accordance with the primary working temperature. At this stage, the primer merges with a specific part of the strand of the DNA molecule that has been separated. The last stage of the DNA amplification process is DNA synthesis, where the temperature is raised again to 76 °C to adjust the temperature for the enzyme to work (Feranisa, 2016). The primer will then form an elongation with the help of the enzyme DNA polymerase and form a new strand. When DNA synthesis is complete, the probe emits a signal from its molecules and is calculated automatically by the machine. The final extension was carried out for 10 minutes to give the enzyme time to complete the chain's elongation (Kurniawati et al., 2019). The amplification process was carried out over as many as 40 cycles, with each cycle consisting of denaturation at 94 °C for 30 seconds, annealing at 64 °C for 60 seconds, and DNA synthesis at 94 °C for 30 seconds. One cycle is said to have passed if the test has passed these three stages (Carter et al., 2020; Helmi, Widayanti, & Haryanto, 2014; Jiang et al., 2017; Rehman et al., 2022).

Avian influenza virus is a virus that has genetic material in the form of RNA; therefore, reverse transcriptase enzymes are used in master mix reagents that convert a single chain of RNA into complementary DNA (Aditama et al., 2012; Haryanto et al., 2010). Synthesizing complementary DNA occurs at 48 °C for 45 minutes or one cycle. This stage is carried out before DNA amplification, which is carried out over as many as 40 cycles (Helmi et al., 2014). Virus detection with real-time PCR techniques depends on the machine's performance, the reagents, and the technicians' expertise during PCR work. Therefore, intensive training for laboratory personnel who perform DNA or RNA extraction is needed to gain expertise and consistency among laboratory technicians so that test results can be trusted and there is no contamination (Hewajuli & Dharmayanti, 2014).

Polymerase chain reaction (PCR) is a highly sensitive and specific technique used in molecular biology to amplify and detect specific DNA sequences. PCR has several advantages in detecting avian influenza virus material, including (1) sensitivity, as PCR can detect extremely low levels of avian influenza virus material, even in samples with very low viral loads. By amplifying the target DNA sequences, PCR can detect and identify the presence of the virus with high sensitivity. (2) Specificity: PCR can distinguish between different strains or subtypes of avian influenza viruses. By designing specific primers that match the unique genetic sequences of the target virus, PCR can provide accurate and specific detection, allowing for the identification of the specific avian influenza strain present. (Musdalifa et al., 2020). (3) Speed, PCR is a rapid technique that can provide results within a few hours. Compared to traditional virus isolation methods, which can take several days, PCR allows for a quicker detection and diagnosis of avian influenza infections. Rapid identification is crucial for implementing timely control measures and preventing further spread of the virus.

(4) Early detection: PCR can detect avian influenza virus material at an early stage of infection, even before clinical signs become apparent in the birds. This early detection enables prompt intervention and control measures to be implemented, reducing the risk of virus spread within the poultry population and to other bird species (Adnyana, 2023). (5) Quantification: Real-time PCR variants, such as quantitative PCR (qPCR), can not only detect the presence of avian influenza virus material but also provide information about the viral load in a sample. This quantitative aspect of PCR allows for monitoring viral replication dynamics and assessing the severity of infection. (6) Versatility: PCR can be used to detect avian influenza virus material in a variety of sample types, including swabs from the respiratory tract, cloacal

swabs, feathers, eggs, or tissues. This flexibility makes PCR a valuable tool for avian influenza surveillance, diagnosis, and research. (7) With automation and high-throughput capabilities, PCR can be easily automated using specialized instruments, allowing for high-throughput testing of multiple samples simultaneously. This automation and scalability make PCR suitable for large-scale screening of avian influenza in commercial poultry farms or during outbreaks, where a large number of samples need to be processed quickly.



**Figure 2.** DNA amplification process with real-time PCR. Information; (a) RNA Extraction Stage Tools and Materials; (b) Adding lysis buffer and proteinase K to microtubes; (c) Incubation Process; (d) Adding Ethanol; (e) Sample centrifuged for 1 minute at 8,000 RPM; (f) Providing Washing Buffers; (g) Controller Mix Process; (h) Creating Templates. (i) PCR Test Ready Samples.

#### 4. CONCLUSION

Real-time polymerase chain reaction (RT-PCR) was used to quantitatively and molecularly detect avian influenza. Its wide range of use is essential to detect food contaminated with the virus to reduce morbidity due to this disease. In laboratory examinations, positive sample samples are shown by graphs that cross the threshold on cycles below 40, while the detection of nominal Ct shows quantitative data. However, the sample used does not have a nominal Ct, and the graph does not cross the threshold, so the sample tested negative for the avian influenza virus. The DNA amplification process of the avian influenza virus begins with an amplification process of 40 cycles, with each cycle stage taking the form of denaturation at 94 °C for 30 seconds, annealing at 64 °C for 60 seconds, and DNA synthesis at 76 °C for 30 seconds. One cycle is declared if the amplification process has passed these three stages. In the future, it will be necessary to identify the accuracy, sensitivity, and specificity of real-time PCR and develop rapid test kits for AI virus detection.

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