

Development of Highly Sensitive Conventional PCR for African Swine Fever Virus Diagnosis in East Nusa Tenggara (NTT) Province

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ABSTRAK

African Swine Fever (ASF) adalah penyakit menular pada babi dengan tingkat mortalitas mencapai 100%. Pada tahun 2019, penyakit ini sebabkan wabah pada provinsi Nusa Tenggara Timur (NTT), dimana merupakan provinsi penghasil babi terbesar di Indonesia. PCR masih digunakan sebagai alat diagnosa untuk deteksi ASF virus (ASFV). Lepas dari sensitifitas dan spesifitasnya mencapai 90%, hasil dari PCR untuk mendeteksi ASFV masih memberikan *false negatives* pada beberapa laboratorium. Sehingga, tujuan dari penelitian ini adalah untuk mengembangkan PCR yang sangat sensitif untuk deteksi ASFV secara akurat di NTT. Metode penelitian dimulai dengan penentuan tipe dari sampel, *primers setup*, ekstraksi DNA, pencampuran *master mix*, proses amplifikasi, dan elektroforesis. Hasil PCR menunjukkan bahwa ASFV dideteksi pada hati, ginjal, dan limpa dari babi yang mati di Kabupaten Kupang, NTT dengan menggunakan primer : 5' CGCAGAGGTAAGCTTTTCAGG 3' (*forward primer*) dan 5' GCCGATACCACAAGATCAGC 3' (*reverse primer*) dari gen p72. Panjang produk PCR mencapai 372 bp. Sehingga, hasil studi ini dapat diaplikasikan sebagai referensi bagi laboratorium di NTT dalam mendiagnosa ASF sehingga penyakit tidak menyebar dengan cepat dan menyebabkan wabah berikutnya.

Kata Kunci : *African Swine Fever*; Kupang; PCR; Primer

INTRODUCTION

The pig population in East Nusa Tenggara (NTT) Province is the largest in Indonesia, reaching 2,6 million in 2021 (Vika, 2021). This number is not surprising because almost 69% of household heads raise pigs as their main income (Leslie et al., 2015). In addition, pigs are the dominant livestock for the cultural

purpose of the NTT community. So, when the African Swine Fever (ASF) outbreak occurred in 2019 until now in NTT, it killed almost 500,000 pigs (Jahang, 2023). The pig deaths disrupt the economic stability of people who rely on pigs as their income.

ASF is caused by the African Swine Fever Virus (ASFV) which is

part of the *Asfarviridae* family (OIE, 2021). This virus has a genome with a length of 170-190 kbp, controlling 68 proteins (Alejo et al., 2018; Dixon et al., 2013). These proteins build the envelope, capsid, inner membrane, core-shell, and genome (Alejo et al., 2018). The recommended genes of ASFV as primers for PCR detection are P72, P54, CD2v, and B602L genes (Shi et al., 2022).

Diagnosing the disease using PCR is an important skill for early detection. PCR is a recommended method by the OIE to detect ASFV since it has high sensitivity and specificity (OIE, 2021). However, if the preparation is not properly performed, the results may give a false negative (Gonzague et al., 2002). Lack of preparation for primers led to false negative results for ASFV in Vietnam (Truong et al., 2020). Based on studies in the field, there are differences in PCR results for ASFV detection conducted among laboratories in NTT. Moreover, one laboratory reported that it was positive, while another said the result

was negative. This is fatal if a false negative result is adopted by the decision-makers to allow pigs to exit from positive areas for ASF into free areas. The quality and clarity of the PCR results rely on many factors, namely the isolation procedure, the appropriate DNA concentration, DNA quality, the primer, and the PCR cycle (Wahyuningsih et al., 2017). In addition, pigs infected with ASFV show clinical symptoms or post mortem changes that are difficult to distinguish from diseases such as Hog Cholera, Erysipelas, and Porcine Reproductive and Respiratory Syndrome (PRSS).

Based on those problems, developing a highly sensitive conventional PCR method is needed. This study aims to explore the proper type of sample, how to design primers, DNA extraction, the mixing process of the master mix, the amplification process, and electrophoresis to detect the ASF virus. So, this method can be used as a reference for ASFV detection in laboratories in NTT.

MATERIAL AND METHOD

The research was conducted at the Molecular Laboratory, Faculty of Medicine and Veterinary Medicine, Universitas Nusa Cendana (UNDANA). No need for ethical clearance because the samples were collected from two dead pigs between February to March 2023 in Kupang District, NTT. The samples were the liver, kidney, and spleen from one

dead pig.

The P72 gene was used to make primers for ASFV detection by modifying the method from our previous study (Pandarangga et al., 2022). The isolate "African swine fever virus isolate Uga12 Lango3 P72 (P72) gene" with accession number KF303321.1 was applied for primer design. All the default parameters

from the “Pick Primers” section were used, except PCR product, T_m Temperature, a database, and organism type. The PCR product range was 80-250 bp with a minimum T_m temperature of 58 °C and the difference in T_m was no more than 5 degrees. For the database, the selection was changed to “Refseq representative genome” with the organism selection being “*Asfarviridae* (taxid: 137992)”. In the advanced parameters section, all the parameters were defaulted except for the primer size GC content, 18-30 bp and 40-60%, respectively. Moreover, using more than the three same nucleotides in the row was not recommended for designing a good primer, for example, ACCCC.

The tissue weight was up to 25 mg. The reagent for the viral DNA extraction was DNeasy Blood & Tissue Kit (QIAGEN, Germany). The procedure was carried out following the kit protocol with a few modifications. Each tissue was chopped finely using blade number 20 in a disposable petri dish, then put into a 1.5 ml tube containing 180 µl of Buffer ATL that was mixed with 20 µl of proteinase K. The mixture was homogenized using a vortex for 30 seconds. The sample was incubated in a thermocycler for 1 hour at 56 °C. During the incubation, the sample was mixed thoroughly every 15 minutes using a vortex. 200 µl Buffer AL was poured into the mixture and then incubated at 56 °C for 10 minutes. 200 µl ethanol was added and then homogenized using a vortex.

The mixture was placed into the DNAease column with a base 2 mL collection tube. This tube was centrifuged at 8000 rpm for 2 minutes. After replacing the collection tube, 500 µl Buffer AW was added into the spin column, and then centrifuged for 2 minutes at 8000 rpm. The same procedure was performed after adding 500 µl Buffer AW2. The collection tube was replaced with a new one and DNA was eluted using 20 µl Buffer AE. Before centrifuging, the buffer was incubated for 1 minute. The DNA template was ready for PCR.

The mixing process was performed according to the protocol of the AllTaq Master Mix Kit (Qiagen, Germany). The final concentration of Alltaq Master Mix was 1 time with 5 µl volume per reaction. Each primer concentration was 10µM with 0.25 µM as the final concentration. As optional, a 0,16 µl master mix tracer with 1-time final concentration was added to the master mix. A template DNA sample was 4 µl. Water was added to the master mix up to 20 µl volume. The master mix was put into a 0.2 mL PCR tube and then centrifuged for 30 seconds. Initial denaturation was at 95 °C for 2 minutes and then followed by 40 cycles of denaturation at 95 °C for 5 seconds; annealing at 55 °C for 15 seconds; and extension at 72 °C for 10 seconds were the setup for DNA amplification.

Identification of PCR product was carried out using an electrophoresis tool (Bio-Rad™

Electrophoresis System) (Pandarangga, 2021). The first step was the preparation of a 2% agarose gel and staining of the PCR product and the ladder. The gel was obtained from 2 grams of agarose by dissolving in 100 mL of TBE 1X using a microwave for 3 minutes or until all the powder dissolved. After a few moments, the liquid agar was poured into the cast with the comb to form wells. When the gel had hardened, it was put into the electrophoresis chamber which was filled with 1X TBE buffer to three-quarters of the chamber space. The next step was the preparation of loading dye to the PCR

product. Each 2 μ l of six times loading dye with gel red nucleic (10,000 times) was mixed into 5 μ l PCR product and 3 μ l ladder. Then, each mixture was poured into a well. In the beginning, the electrophoresis machine was set up at 100 Volts for the first 5 minutes and then continued at 90 Volts for 60 minutes. The results were visualized with a UV transilluminator machine and photographed using a cell phone camera. Each sample with DNA bands was compared with a 100 bp DNA Ladder (Invitrogen) to determine the length of the PCR products.

RESULTS AND DISCUSSION

The samples, such as liver, kidney, and spleen collected from pig deaths in Kupang Regency, NTT, Indonesia, were positive for ASFV. The primer was created from the p72 gene of the virus isolate Uga12 Lango3 at the molecular laboratory of the Study Program of Veterinary Medicine, UNDANA. P 72 gene was one of the gene recommendations to detect ASFV in PCR (Shi *et al.*, 2022). This primer was named PP/2022/ASFV, consisting of 5' CGCAGAGGTAAGCTTTCAGG 3' as forward primer and 5' GCCGATACCACAAGATCAGC 3' as reverse primer. The PCR product was 372 bp. Among the three organs tested, only the kidney showed a thinner PCR product band compared to the band from the liver and spleen. The results are depicted in Figure 1.

The ASFV target is monocytes and macrophages, which in the liver, kidney, and lung are known for local mononuclear phagocyte system (MPS) cells (Franzoni *et al.*, 2023). So, DNA fragments of the virus were presented into the bands in agarose gel, shown in all samples. However, the PCR product band for the kidney was thinner compared to others. This is because only a small amount of the virus goes to the kidneys (Aguero *et al.*, 2003). Additional evidence using the immunohistochemical method shows very low ASFV viral loading in the kidneys (Izzati *et al.*, 2021). The PCR product band for the liver sample was thick. This is because of the large number of Kupffer cells available as ASFV target cells (Blome *et al.*, 2020). The PCR band for the spleen was thick (Okwasiimire *et al.*,

2023). This is because the spleen has abundant macrophages and monocytes. So, it is no surprise that the spleen showed severe

hemorrhagic, fibrin deposition, and cell damage during ASFV infection (Zhu et al., 2019).

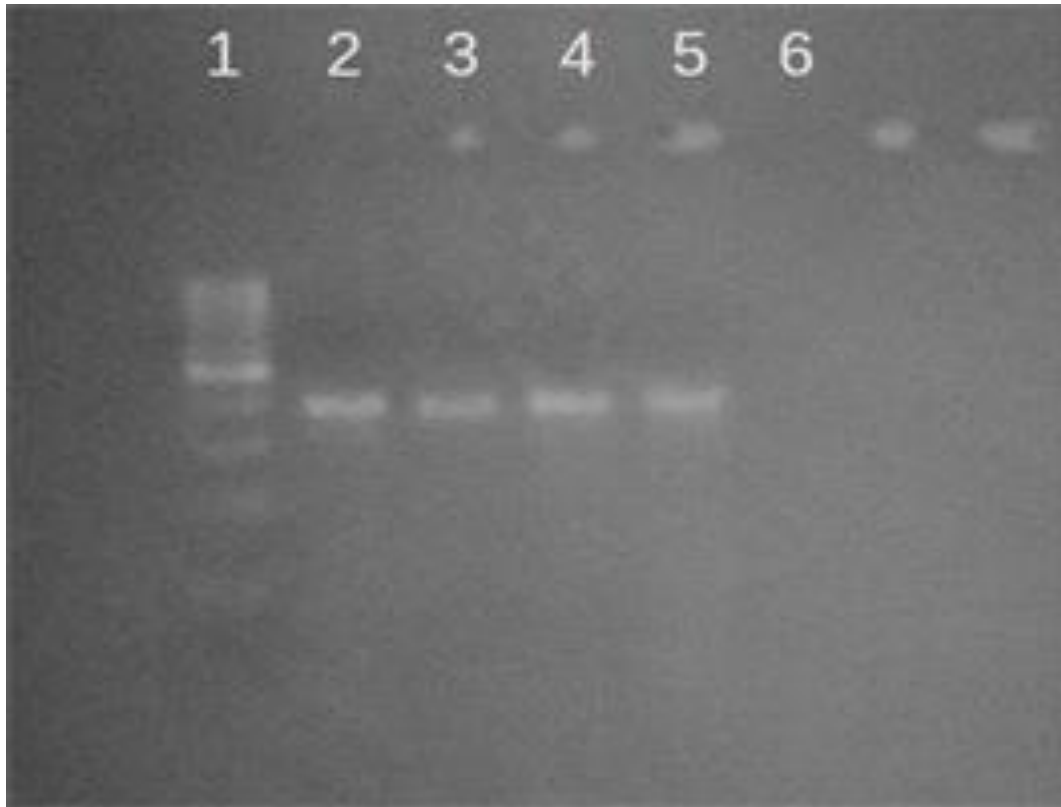


Figure 1. PCR test detection results using PP/2022/ASFV primer. 1=100 bp ladder (Thermo Scientific GeneRuler); 2=Liver; 3=Kidney; 4=Liver; 5=Spleen; 6=Negative Control

In this study, the liver and spleen samples displayed the same thickness of PCR band which represents the amount of DNA fragments from ASFV. However, for practical reason, the spleen is not recommended in this study because the reticular connective tissue or stroma clog the filter tube during the DNA extraction process even though it has been lysed. So, the liver is the proper organ for ASFV detection. By applying a liver sample from one dead pig in Kupang District, we performed

a whole genome sequence (WGS) for ASFV (under review process) using the method of our previous study in analyzing WGS for the Newcastle Disease Virus (Doan et al., n.d.; Pandarangga et al., 2020). The sequences have been registered into NCBI with accession numbers OR604566 and OR604567.

Moreover, a lack of preparation and extraction procedures will produce false negatives due to the presence of inhibitors (Gonzague et al., 2002). Decisions based on the

false negative results to allow pig movement from ASF-infected areas to ASF-free areas may result in new ASF outbreaks. So, in this study, we follow the protocol instructions carefully and modify them based on the available resources without

violating the procedures. This method can be applied as a reference regarding the organ type and the procedures for PCR testing in laboratories in NTT to prevent further ASFV outbreaks.

CONCLUSION

The PP/2022/ASFV primer, which comes from the p72 gene with a product length of 372 bp, has succeeded in detecting the ASFV presence in the liver, spleen, and kidney from dead pigs in Kupang

District. Among the positive samples, the liver is the best sample used for ASFV identification in pigs and also for further analysis such as whole genome sequences analysis.

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