CHAPTER IV
RESULTS AND DISCUSSION

4.1 Detection of *orf2* and *orf11* of T3SS-1 of *B. pseudomallei* by polymerase chain reaction (PCR) amplification

The target sites of the present study were the open reading frames: *orf2* and *orf11* from T3SS-1 gene cluster that were the specific markers of *B. pseudomallei* distinct from another closely related species, *B. thailandensis* and *B. mallei* (Chantratita et al., 2008; Novak et al., 2006; Thibault et al., 2004). The genomic DNA of the wild type *B. pseudomallei* strain K96243 and isolates of *B. pseudomallei* from clinical samples were extracted by boiling method according to Armed and coworker, 2017. The extraction was performed with precipitation and without precipitation by absolute ethanol, followed by detection of *orf2* and *orf11* using the PCR technique. The PCR products of *orf2* and *orf11* were detected as 250 bp and 335 bp, respectively using 2% agarose gel electrophoresis. In this study, the genomic DNA of *B. pseudomallei*, Gram-negative bacteria, was extracted using a modified boiling method for rapid extraction, specifically for the purposes of PCR and CRISPR/Cas12a analysis. In addition, alcohol precipitation is commonly used for concentrating, desalting and recovering nucleic acids which precipitation is mediated by the addition of ethanol.

In the present study, both the wild type *B. pseudomallei* strain K96243 and clinical isolates of *B. pseudomallei* exhibited identical PCR products of *orf2* and *orf11* which were detected at 250 bp and 335 bp, respectively as shown in the Figure 4.1, 4.2 and 4.3. The results suggested that the primers designed for *orf2* and *orf11* in this study could be useful for investigation *B. pseudomallei* strain found in clinical samples. Furthermore, the results of this study demonstrated that DNA extracted by boiling method without ethanol precipitation appeared as a clear single band in the agarose gel similar to DNA extracted by boiling method with ethanol precipitation and DNA

extracted by boiling method with ethanol precipitation and incubated overnight at -20 ℃, which indicates that DNA was not degraded and provided the sufficient DNA yield. Hence, the boiling method offers a rapid, easy and cost-effective approach for high-yield DNA isolation from gram-negative bacteria. Additionally, this method circumvent the need for toxic chemicals.

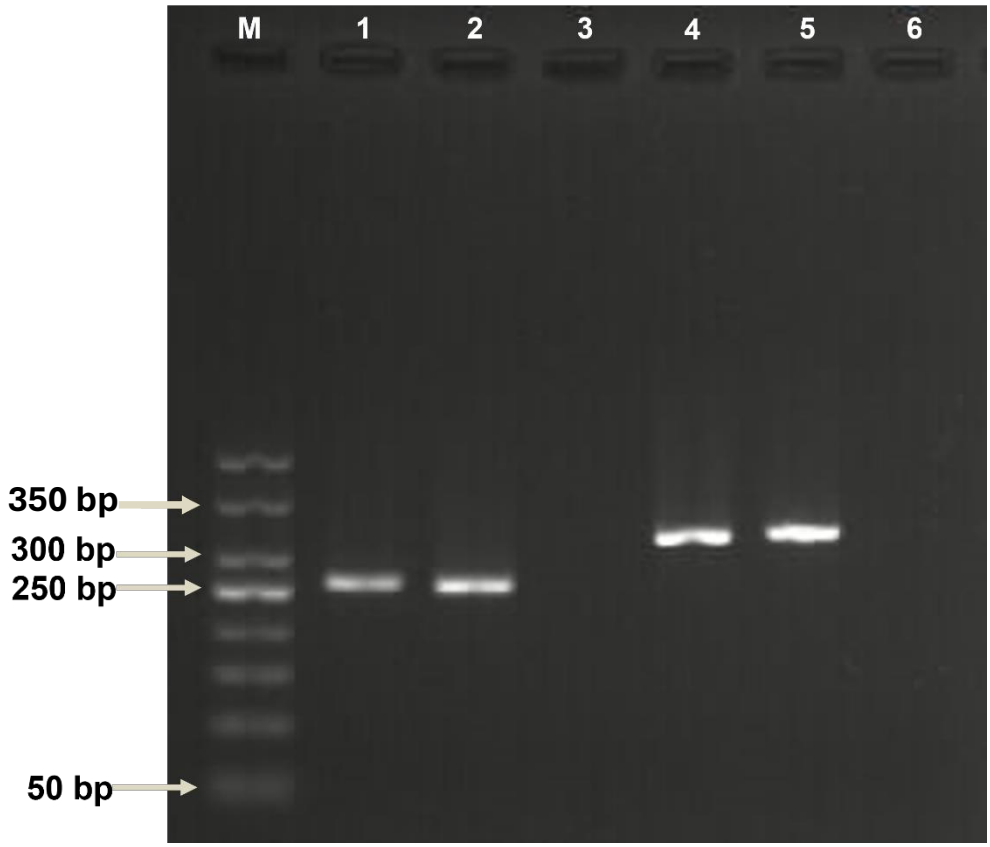

Figure 4.1 The PCR products of *orf2* and *orf11* from T3SS-1 of the wild type *B. pseudomallei* strain K96243 exhibited 250 bp and 335 bp, respectively using 2% agarose gel electrophoresis. Lane 1: PCR products of *orf2* which DNA extracted by boiling method with ethanol precipitation, Lane 2: PCR products of *orf2* which DNA extracted by boiling method without ethanol precipitation, Lane 3: negative control for *orf2*, Lane 4: PCR products of *orf11* DNA extracted by boiling method with ethanol precipitation, Lane 5: PCR products of *orf11* DNA extracted by boiling method with ethanol precipitation, Lane 6: negative control for *orf11*, Lane M:50-bp DNA ladder.

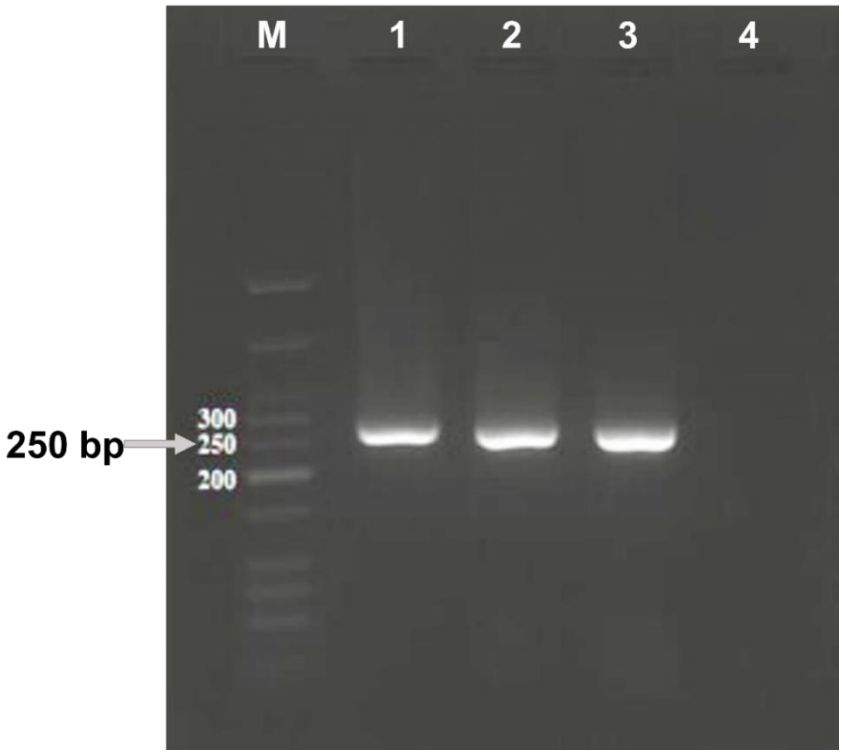

Figure 4.2 The PCR products of *orf2* from T3SS-1 of clinical isolate *B. pseudomallei* exhibited 250 bp using 2% agarose gel electrophoresis. Lane 1: DNA extracted by boiling method with ethanol precipitation, Lane 2: DNA extracted by boiling method without ethanol precipitation, Lane 3: DNA extracted by boiling method with ethanol precipitation and incubated overnight at -20 ℃, Lane 4: negative control Lane M: 25-bp DNA ladder.

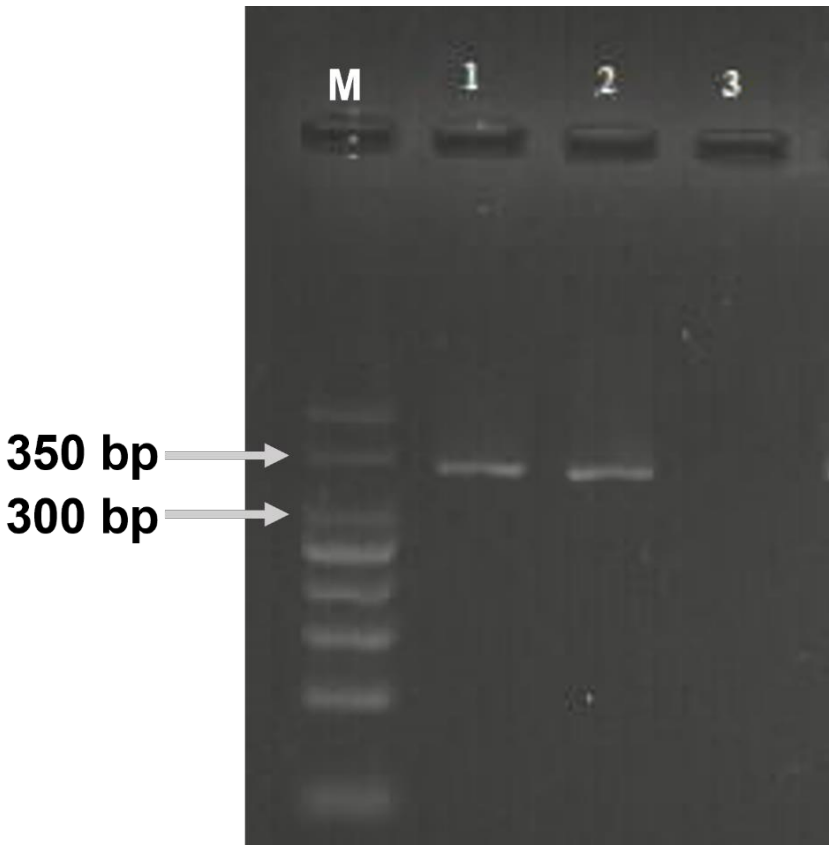

Figure 4.3 The PCR products of *orf11* from T3SS-1 of clinical isolate *B. pseudomallei* exhibited 335 bp using 2% agarose gel electrophoresis. Lane 1: DNA extracted by boiling method with ethanol precipitation, Lane 2: DNA extracted by boiling method with ethanol precipitation, Lane 3: negative control, Lane M: 50-bp DNA ladder.

4.2 Determination of the specificity of selected target site *orf2* and *orf11* from T3SS-1 *B. pseudomallei* with another Gram-negative bacilli

The soil saprophyte *B. thailandensis* is non-pathogenic for humans and animals and present abundant in the soils and standing waters of endemic areas. This species is closely related to *B. pseudomallei* and *B. mallei* as its genome encodes numerous homologs of virulence factors from these pathogenic species (Broek and Stevens, 2017; Thibault et al., 2004). In addition, *B. thailandensis* displays phenotypic characteristics that make it appear similar to *B. pseudomallei* by routine diagnosis tests. The *B. pseudomallei* genome encodes three T3SS which are referred to as T3SS-1, T3SS-2

and T3SS-3. The genomes of *B. pseudomallei* consists of two circular chromosomes., with all three T3SS residing on chromosome 2. T3SS-2 and T3SS-3 are present in the genomes of *B. mallei* and *B. thailandensis*, whereas T3SS-1 is absent from both (Broek and Stevens, 2017; Chantratita et al., 2008; Novak et al., 2006; Thibault et al., 2004). In the previous studies also reported that a 548-bp region of T3SS-1 of *B. pseudomallei* encompassing part of *orf2* was found to be present in *B. pseudomallei* and but not in related *B. mallei* or *B. thailandensis* (Novak et al., 2006). Furthermore, Thibault and co-worker also reported that *orf11* of T3SS-1 is a specific markers of *B. pseudomallei* distinct from other closely related species, *B. thailandensis* and *B. mallei*. Therefore, in this study the isolates of *B. thailandensis* obtained from Melioidosis Center, Faculty of Medicine, Khon Kaen University were used to determine the specificity of *orf2* and *orf11* from T3SS-1 of *B. pseudomallei* using PCR technique. The result revealed that none of the DNA fragments was observed when amplifying *orf11* from *B. thailandensis* by PCR technique as shown in Figure 4.5. Although a slightly intense DNA fragment of *orf2* was observed on the agarose gel, it exhibited a different fragment length from that of *B. pseudomallei* (250 bp), which was used as a positive control as shown in Figure 4.4. The result of *orf11* by PCR technique is consistent with the previous study indicated that *orf11* is a specific marker for *B. pseudomallei* (Thibault et al., 2004) and can be the specific target site for molecular diagnosis such as CRISPR/Cas12a. The finding of *orf2* fragment of *B. thailandensis* on the agarose gel should be improved by sequencing. However, the primers designed of *orf 2* in this study was able to detected *B. pseudomallei* at the DNA fragment length of 250 bp. Subsequently, the PCR products of *orf2* from *B. thailandensis* were tested in CRISPR/Cas12a system to determine the specificity of the designed gRNA used in this study.

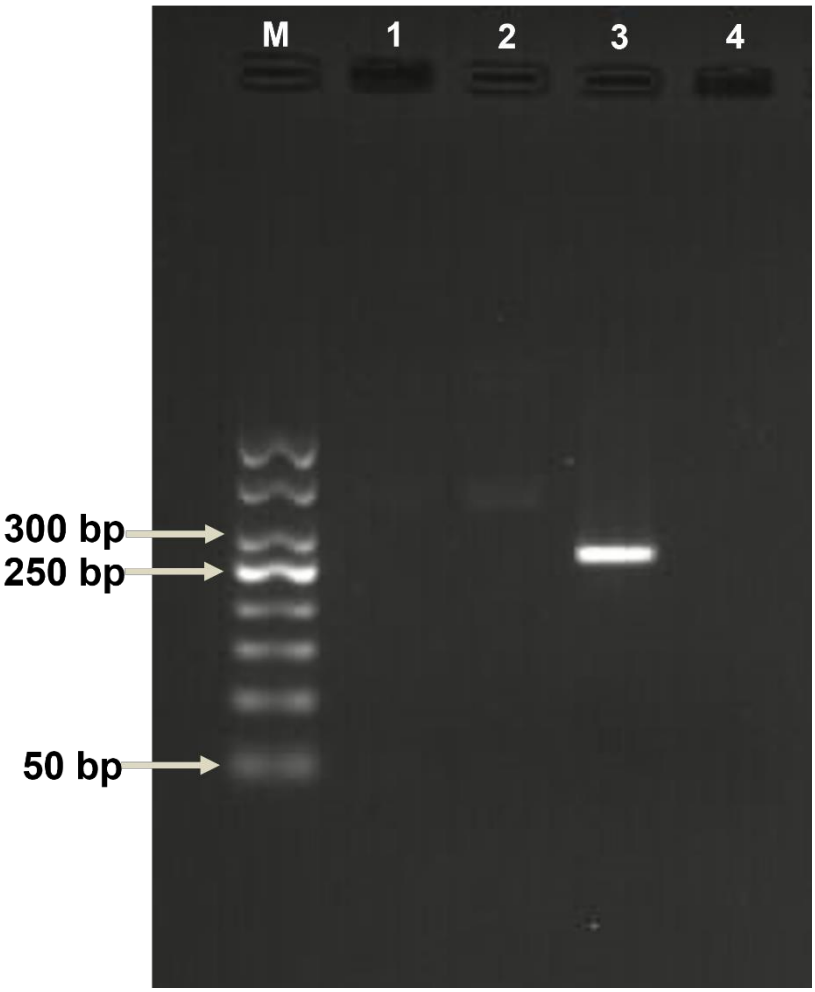

Figure 4.4 The PCR result of amplifying *orf2* from *B. thailandensis* was analyzed by 2% agarose gel electrophoresis to determine the specificity of *orf2* from T3SS-1 of *B. pseudomallei.* Lane 1: extracted DNA of *B. thailandensis* by boiling method with ethanol precipitation, Lane 2: extracted DNA of *B. thailandensis* by boiling method without ethanol precipitation, Lane 3: DNA of *B. pseudomallei* as a positive control (250 bp), Lane 4: negative control Lane M: 50-bp DNA ladder.

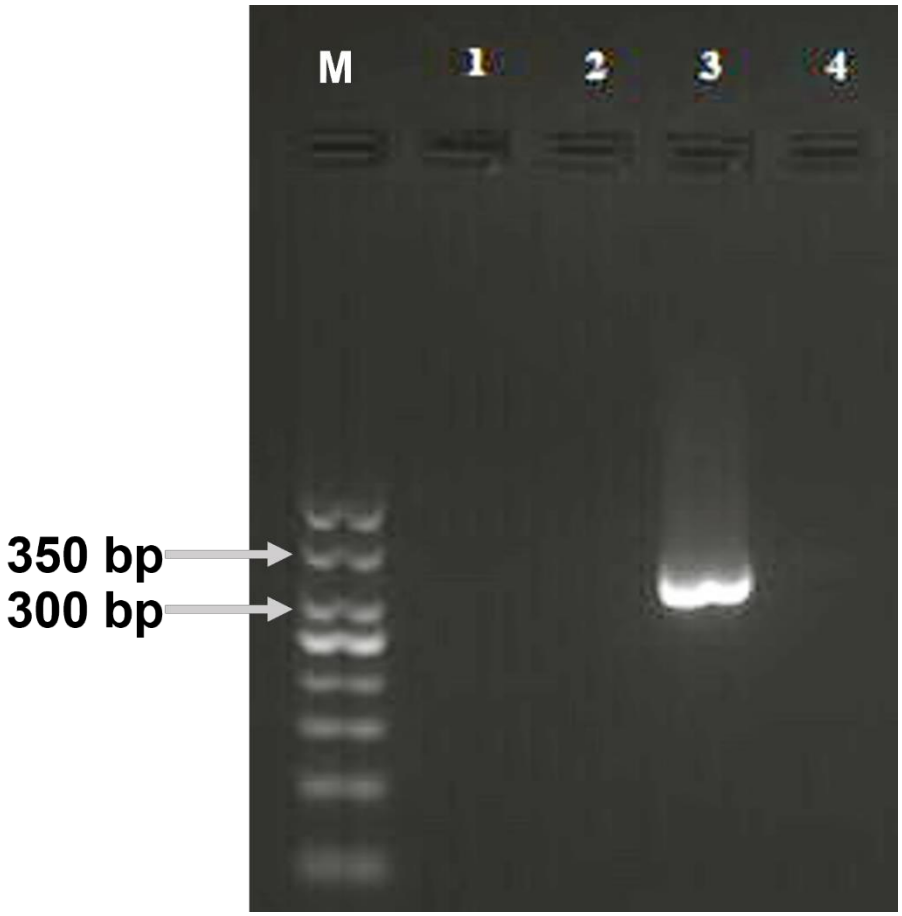

Figure 4.5 The PCR result of amplifying *orf11* from *B. thailandensis* was analyzed by 2% agarose gel electrophoresis to determine the specificity of *orf11* from T3SS-1 of *B. pseudomallei.* Lane 1: extracted DNA of *B. thailandensis* by boiling method with ethanol precipitation, Lane 2: extracted DNA of *B. thailandensis* by boiling method without ethanol precipitation, Lane 3: DNA of *B. pseudomallei* as a positive control (335 bp), Lane 4: negative control Lane M: 50-bp DNA ladder.

4.3 *In vitro* digestion activity of Lba Cas12a (Cpf1) on *B. pseudomallei* DNA target

The specificity of the designed gRNA to *B. pseudomallei* DNA target was tested using the PCR product of *orf2* and *orf11* from T3SS-1 of *B. pseudomallei* containing the target sequence. The designed gRNA of this study binding to target sequences of *orf2* and *orf11* were illustrated in Figure 4.6 and Figure 4.7, respectively. According to

previous studies, Cas12a quickly recognized and cleaved the DNA target site under the guidance of its specific gRNA (Moa et al., 2022). In our study, the *in vitro* digestion activity of RNA-guided enzyme Cas12a or CRISPR/Cas12a on target sites were investigated by 2.5% agarose gel electrophoresis. The results showed that the DNA fragments of *orf2* from both the wild type *B. pseudomallei* strain K96243 and the clinical isolate *B. pseudomallei* exhibited a larger size (350 bp) than its PCR product (250 bp) which were used as a control after adding gRNA and Cas12a into the reaction. Additionally, a smaller fragment of more than 50 bp was also observed (Figure 4.8 and Figure 4.9). Conversely, the DNA targets of *orf11* from both the wild type *B. pseudomallei* strain K96243 and the clinical isolate *B. pseudomallei* were cleaved into fragments ranging between 150 bp and 200 bp which were smaller than PCR product used as control (335 bp), as shown in Figure 4.8 and Figure 4.10. In theory, if the digestion occurs in the reaction involving the gRNA and Cas12a enzyme, it should have generated small sizes of the DNA fragments. However, in our study the *orf2* reaction showed 3 distinct bands, one of which showed a larger size of fragment band. This could be explained by the presence of tightly bound proteins (endonuclease enzyme) and nucleic acid (DNA) which form stable complexes during gel electrophoresis. The presence of complexes can cause slow-moving bands of DNA. This finding might further explain the efficiency of the gRNA of *orf2* and suggest that the gRNA may not be able to completely induce the digestion activity by Cas12a. Therefore, it might not be the best selected gRNA for the development of a diagnostic tool for a test with good sensitivity.

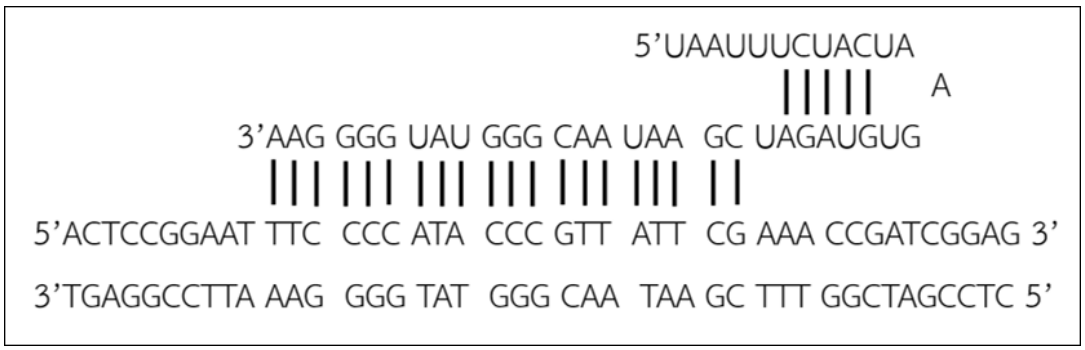

Figure 4.6 The gRNA binding to target sequences of *orf2* from T3SS-1 of *B. pseudomallei*.

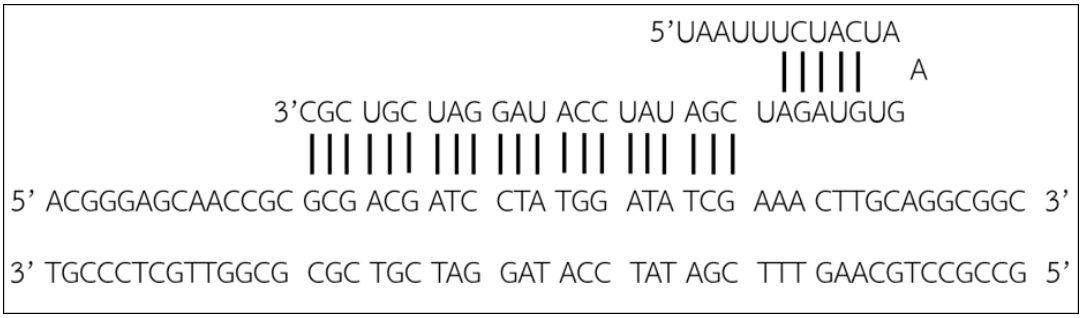

Figure 4.7 The gRNA binding to target sequences of *orf11* from T3SS-1 of *B. pseudomallei*.

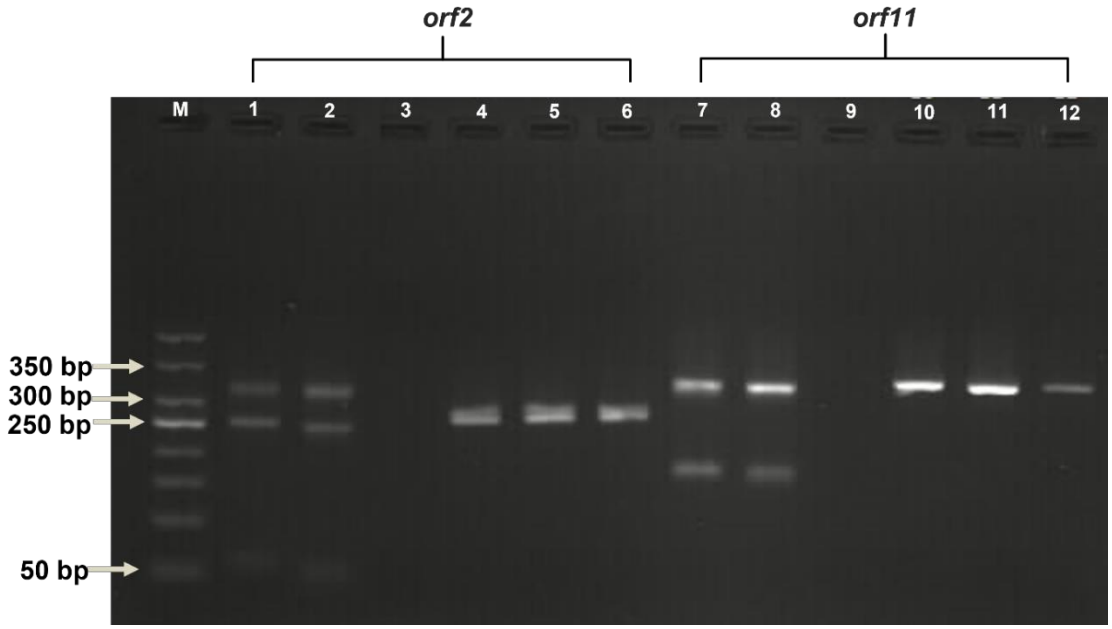

Figure 4.8 *In vitro* digestion activity of RNA-guided enzyme Cas12a or CRISPR/Cas12a on target sites of *orf2* and *orf11* from T3SS-1 of the wild type *B. pseudomallei* strain K96243 were investigated by electrophoresis with 2.5% agarose gel. Lane 1: DNA fragments of *orf2* exhibited different fragment sizes of 250, 350 and >50 bp after adding RNA-guided enzyme Cas12a (genomic DNA was extracted by boiling method with precipitation), Lane 2: DNA fragments exhibited different fragment sizes of 250, 350 and >50 bp after adding RNA-guided enzyme Cas12a (genomic DNA was extracted by boiling method without precipitation), Lane 3: gRNA and Cas12a without PCR product, Lane 4: PCR product and Cas12a except gRNA (genomic DNA was extracted by boiling method with precipitation), Lane 5: PCR product and Cas12a except gRNA (genomic DNA was extracted by boiling method without precipitation), Lane 6: PCR product, Lane 7: DNA fragments of *orf11* exhibited smaller size (>150 bp) than PCR product (335 bp) after adding RNA-guided enzyme Cas12a (genomic DNA was extracted by boiling method with precipitation), Lane 8: DNA fragments of *orf11* exhibited smaller size (>150 bp) than PCR product (335 bp) after adding RNA-guided enzyme Cas12a (genomic DNA was extracted by boiling method without precipitation), Lane 9: gRNA and Cas12a without PCR product, Lane 10: PCR product and Cas12a except gRNA (genomic DNA was extracted by boiling method with precipitation), Lane 11: PCR product and Cas12a

except gRNA (genomic DNA was extracted by boiling method without precipitation), Lane 12: PCR product, Lane M: 50-bp DNA ladder.

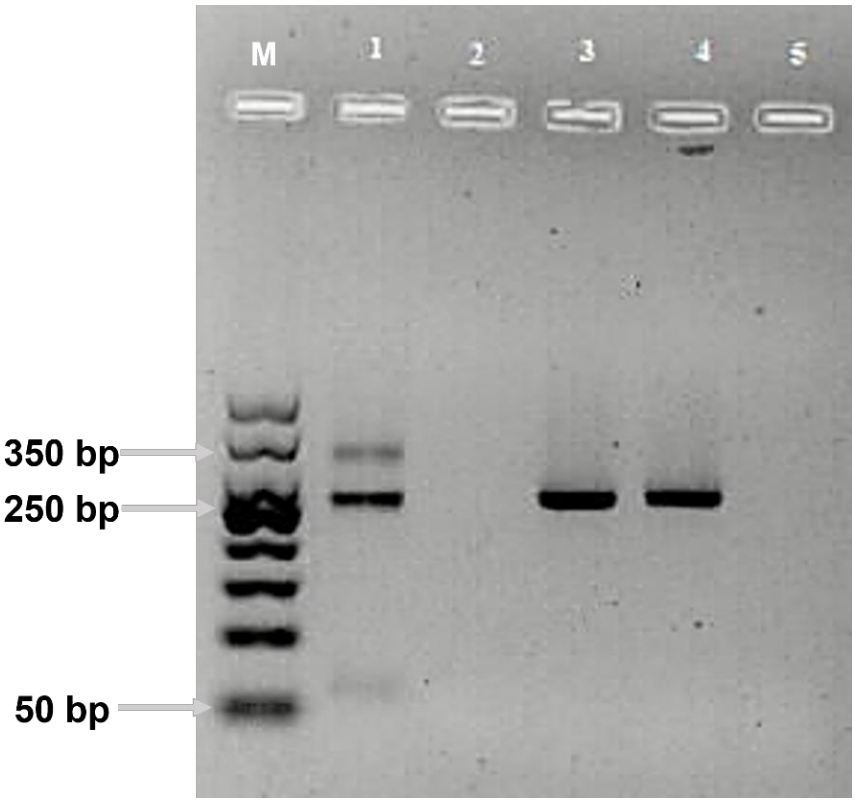

Figure 4.9 *In vitro* digestion activity of RNA-guided enzyme Cas12a or CRISPR/Cas12a on target sites of *orf2* from T3SS-1 of clinical isolate *B. pseudomallei* were investigated by electrophoresis with 2.5% agarose gel. Lane 1: DNA fragments exhibited different fragment sizes of 250, 350 and >50 bp after adding RNA-guided enzyme Cas12a, Lane 2: gRNA and Cas12a without PCR product, Lane 3: PCR product and Cas12a without gRNA, Lane 4: PCR product, Lane 5: negative control, Lane M: 50-bp DNA ladder.

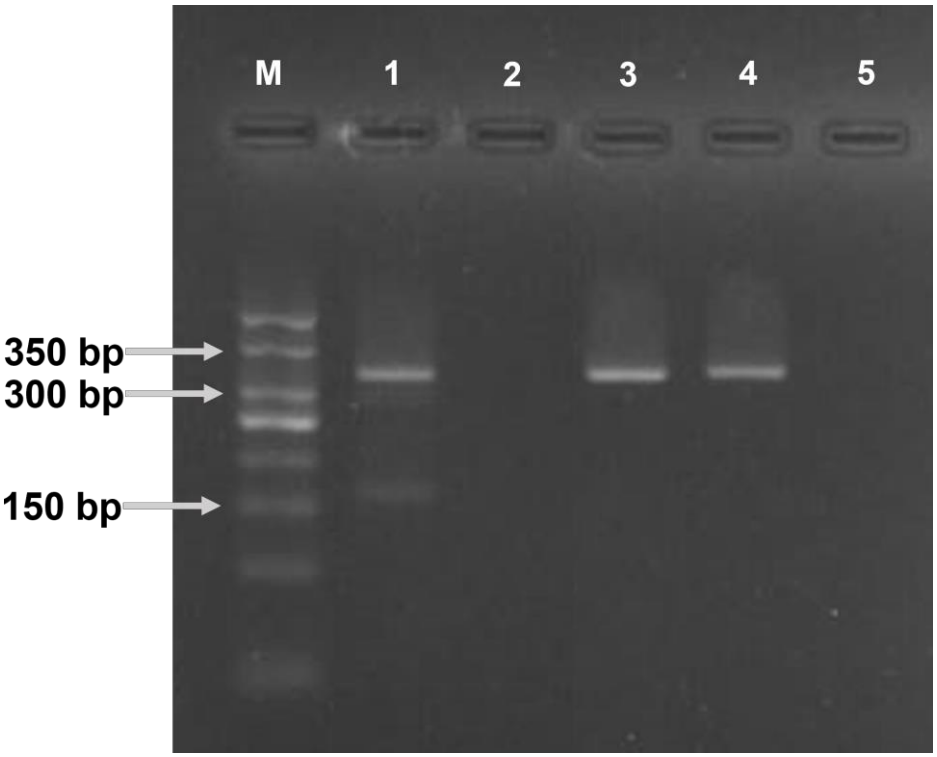

Figure 4.10 *In vitro* digestion activity of RNA-guided enzyme Cas12a or CRISPR/Cas12a on target sites of *orf11* from T3SS-1 of clinical isolate *B. pseudomallei* were investigated by electrophoresis with 2.5% agarose gel. Lane 1: DNA fragments exhibited smaller size (>150 bp) than PCR product (335 bp) after adding RNA-guided enzyme Cas12a, Lane 2: gRNA and Cas12a without PCR product, Lane 3: PCR product and Cas12a without gRNA, Lane 4: PCR product, Lane 5: negative control, Lane M: 50-bp DNA ladder.

4.4 Measurement the signal of single stranded DNA fluorophore-quencher (ssDNA-FQ reporter) of FAM-BHQ1 in CRISPR/Cas12a system

As Cas12a is considered to have a powerful non-specific cleavage activity to ssDNA after binding to the target DNA. Hence, this study further measured the digestion activity of CRISPR/Cas12a at the DNA target of *B. pseudomallei* by evaluation the signal of ssDNA-FQ reporter carrying fluorescein reporter molecule attached at 5′ end and black hole quencher1 on the 3′ end (FAM-BHQ1). The output fluorescence signal and its intensity are directly related to the presence and concentration of the activated

Cas12a in the reaction system (Xiong, 2020). So, the signal of fluorophore of the cleavage products was monitored every single min until 2 h compared with the fluorescence signal of control groups. The positive signal of fluorescence indicates that RNA-guided enzyme designed in this study specifically reacted with *B. pseudomallei* DNA target in CRISPR/Cas12a system as well as collateral cleavage nearby ssDNA-FQ reporters.

In our study, the target-activated CRISPR/Cas12a cleavage activity was verified based on signal amplification of ssDNA-FQ reporter. The result revealed that an increase of collateral cleavage activity of the FAM fluorophore from its quencher, leading to an increased generation of fluorescence signal, which was observed in both *orf2* and *orf11* of the wild type *B. pseudomallei* strain K96243 and *B. pseudomallei* isolated from clinical sample but not in *B. thailandensis* and control samples (Figure 4.11 and Figure 4.12). Although, the finding of slightly intense *orf2* fragment of *B. thailandensis* on the agarose gel by PCR, further analysis using the CRISPR/Cas12a system with ssDNA-FQ reporter demonstrated that the designed gRNA for *orf2* from T3SS-1 of *B. pseudomallei* of this study was specific for detecting *B. pseudomallei* (Figure 4.11). Furthermore, *B. thailandensis* was not detected using the PCR technique, indicating that *orf11* served as a specific marker for *B. pseudomallei*. Further analysis using the CRISPR/Cas12a system with an ssDNA-FQ reporter demonstrated that the designed gRNA targeting *orf11* from T3SS-1 was specific for *B. pseudomallei* detection (Figure 4.12). These indicated that both gRNA of *orf2* and *orf11* designed in this study could specifically detect *B. pseudomallei*, but not other pathogens.

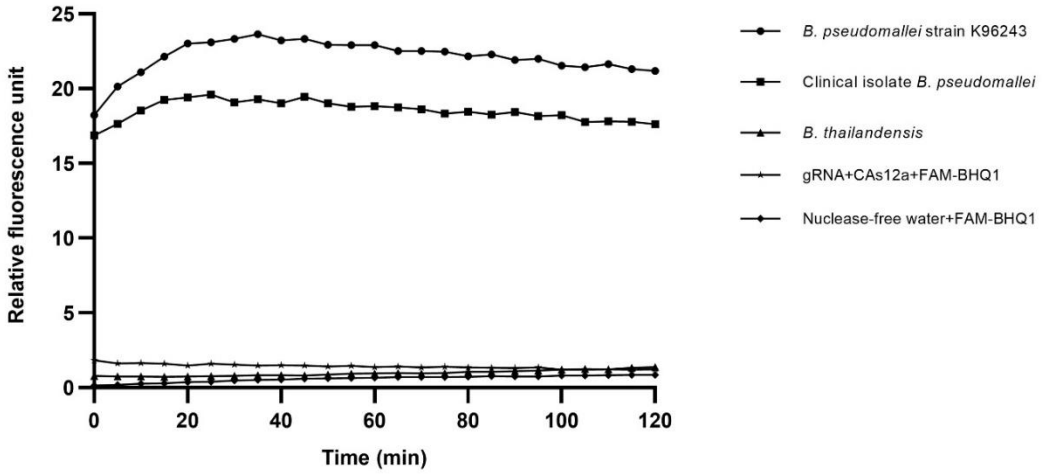

Figure 4.11 The signal of FAM fluorophore of cleavage products of *orf2* from T3SS-1 of *B. pseudomallei* comparing with the fluorescence signals of control groups.

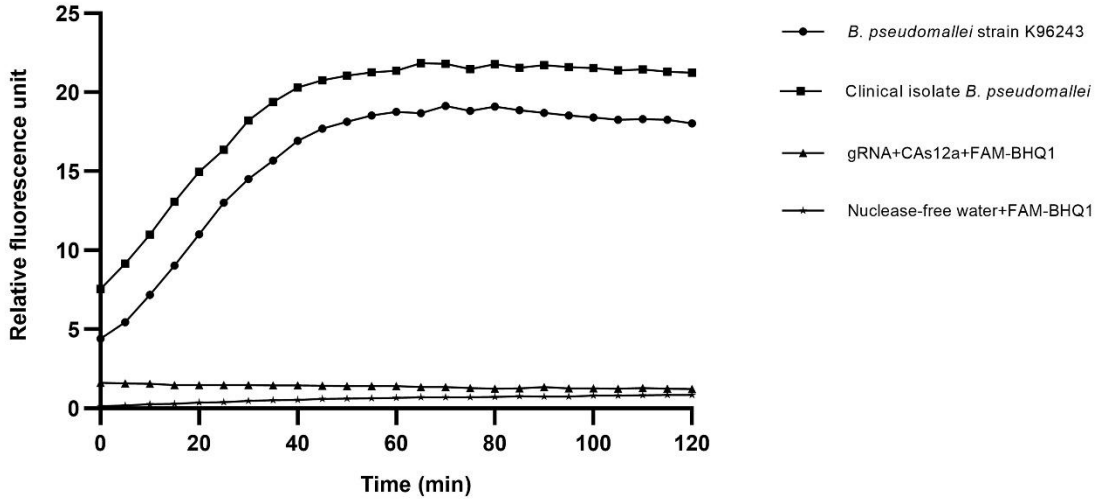

Figure 4.12 The signal of of FAM fluorophore of cleavage products of *orf11* from T3SS-1 of *B. pseudomallei* comparing with the fluorescence signals of control groups.