CHAPTER II

LITERATURE REVIEW

2.1 Melioidosis

Melioidosis is an infectious disease caused by the bacterium *Burkholderia pseudomallei* (*B. pseudomallei*). The disease is most common during the wet season or after major weather events including tropical storms and is most typically related to an inoculating injury through skin, ingestion, or inhalation of dispersed bacteria (Gassiep et al., 2020).

2.1.1 Epidemiology of Melioidosis

Approximately 46 nations have endemic melioidosis, and further 33 nations might contain endemic cases additionally, while autochthonous cases have not yet been reported in these countries (Gassiep et al., 2020). The endemic is high in Northern Australia, Southeast Asia, particularly in Thailand the incidence of melioidosis is still high in Northeast Thailand. Patients with diabetes mellitus, chronic kidney disease, binge alcohol consumption, cystic fibrosis and rice paddy workers are one of the highest risk groups for contracting melioidosis (Selvam et al., 2021). *B. pseudomallei* is found in a wide range of ecological niches, including soil and surface water, and has also been found to adhere to the roots of legumes (Duangurai et al., 2018). A study performed in Northeast Thailand between 1987 and 1991 suggested an incidence of 4.4 cases per 100,000 population per year. More recent observations demonstrated a peak incidence of 21.3 per 100,000 population in 2006 and an average of 12.7 per year for the period from 1997 to 2006 (Gassiep et al., 2020).

2.1.2 Clinical presentation

Melioidosis has been dubbed “the Great Imitator” due to the absence of a clinical syndrome and the ability to exhibit clinical manifestations that mimic other diseases, such as cancer, tuberculosis and other severe infections or pneumonia from another pathogen (Hemarajata et al., 2016). The similarity characterised to tuberculosis and leptospirosis by fever, headache, myalgia, mild or productive cough. Pulmonary melioidosis is the most common clinical presentation of melioidosis where the patient may have features mimicking tuberculosis. It varies from mild to overwhelming necrotizing pneumonia. In humans with melioidosis, the symptoms range from asymptomatic to focal infections such as pneumonia or organ abscesses and systemic diseases (Lee et al., 2010). The disease could be chronic or fulminant with rapidly fatal septicemia, which is often associated with bacterial dissemination to distant sites such as the lungs, liver, and spleen (Figure 2.1). Death can occur in infected humans within 48 h of symptom onset (Novak et al., 2006). In addition, *B. pseudomallei* can survive inside a variety of host cells can resistant to several antibiotics used in the empirical treatment of sepsis, making it difficult to treat, and currently, there is no vaccine to protect against melioidosis.

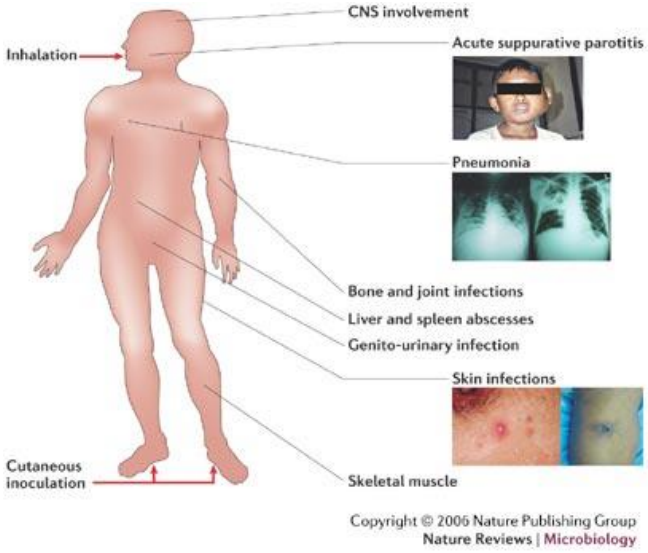

Figure 2.1 Clinical presentations of melioidosis (Wiersinga et al., 2006).

2.2 *Burkholderia pseudomallei*

B. pseudomallei was first discovered in 1911 by Whitmore and his team. This bacterium has been proven to cause melioidosis (Duangurai et al., 2018). *B. pseudomallei* is a Gram-negative, motile, environmental bacterium that appears as small Gram-negative bacilli with bipolar staining, giving them a safety pin appearance (Figure 2.2) (Gassiep et al., 2020). While the genus Burkholderia contains more than 30 species, the most pathogenic members of which are *B. pseudomallei*, *B. mallei*, *B. cepacia* complex, and *B. gladioli*, are generally recognized as human pathogens. These organisms are aerobic, non-spore-forming, nonfermenting Gram-negative bacilli. All are environmental organisms, with the exception of the host-adapted pathogen, *B. mallei*.

B. pseudomallei is found in a wide range of ecological niches, including soil and surface water, and has also been found to adhere to the roots of legumes. The wide variety of *B. pseudomallei* habitats may help explain the persistence of this bacterium in endemic areas (Duangurai et al., 2018). The routes of transmission of *B. pseudomallei* include inoculation via skin abrasion, ingestion, or inhalation of aerosolized bacteria, and ingestion. *B. pseudomallei* measures 2–5 μm in length and 0.4–0.8 μm in diameter and is capable of self-propulsion using flagella. The bacteria are not fastidious and grow on a large variety of culture media (blood agar, MacConkey agar, EMB, etc.). Ashdown's medium is (or *Burkholderia cepacia* medium) used for selective isolation. Cultures typically become positive in 24 to 48 hours (this rapid growth rate differentiates the organism from *B. mallei*, which typically takes a minimum of 72 hours to grow). Colonies becoming dry and wrinkled after 2 days of incubation (Figure 2.3), and present a metallic appearance, and possess an earth odor.

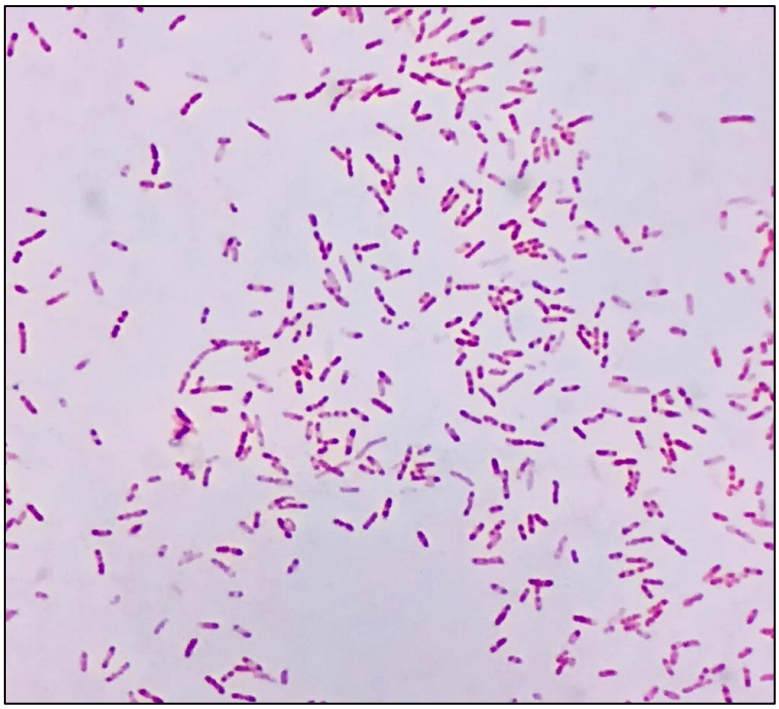

Figure 2.2 Gram stain demonstrating "safety pin" appearance of *B. pseudomallei* Magnification, x100.

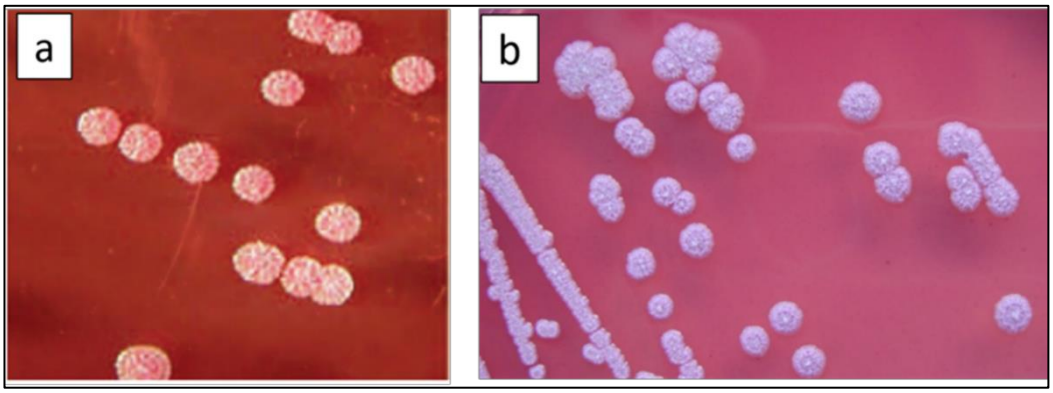

Figure 2.3 Colony morphology of *B. pseudomallei* on blood agar (a) and MacConkey agar (b) (Wiersinga et al., 2006).

2.2.1 Virulence factors of *B. pseudomallei*

B. pseudomallei has several vilurence factors including the cytotoxin *Burkholderia* lethal factor 1 (BLF1), capsular polysaccharide I, the cluster I type VI secretion system (T6SS), the Bsa type III secretion system cluster 3 (T3SS) and Type IV

pili-mediated adherence. BLF1 inhibits translation initiation and subsequent protein synthesis, translation initiation by inactivation of eukaryotic initiation translation factor 4A causing deamidation of glutamine residue (Gln-339) (Rust et al., 2018). Capsular polysaccharide I containing two separate and chemically distinct antigenic O polysaccharides against which infected patients produced antibodies. T6SS which can inject toxins and other effectors into eukaryotic cells. T3SS can secrete effector proteins into the target-cell cytosol to subvert host-cell processes. Type IV pili-mediated adherence is an important virulence mechanism mediated by carbohydrate molecules, pilus and non-pilus adhesins (Bzdyl et al., 2022).

Therefore, *B. pseudomallei* intra and intercellular life cycles can invade cells, the bacteria can invade and propagate in both phagocytic and non-phagocytic cells. These bacteria replicate intracellularly, causing lysis or spreading and infection of adjacent cells. Initially, the bacteria attach to nonphagocytic host cells via flagella, type 4 pili, and adhesins BoaA and BoaB. Cellular invasion is facilitated by the T3SS, which injects effector proteins, including BopA, BopE, BipB, BipC, and BipD. During internalization, the bacterium is enveloped by the host cell in an endocytic vesicle or endosome. Survival within the endosome occurs via multiple processes, including the production of a protease inhibitor, Ecotin. Escape from the endosome is mediated by the T3SS and subsequent upregulation of biosynthesis pathways, including purine, histidine, fatty acid, and amino acid, which aid in replication within the cytosol (Gassiep et al., 2020). Intercellular spread of *B. pseudomallei* is facilitated by membranous protrusions formed by the host cell that extend into neighbouring cells, through which *B. pseudomallei* travel by actin-mediated motility (Wiersinga et al., 2018b) *B. pseudomallei* is able to polymerize actin, spread from cell to cell, causing cell fusion and the formation of multinucleated giant cells (Figure 2.4).

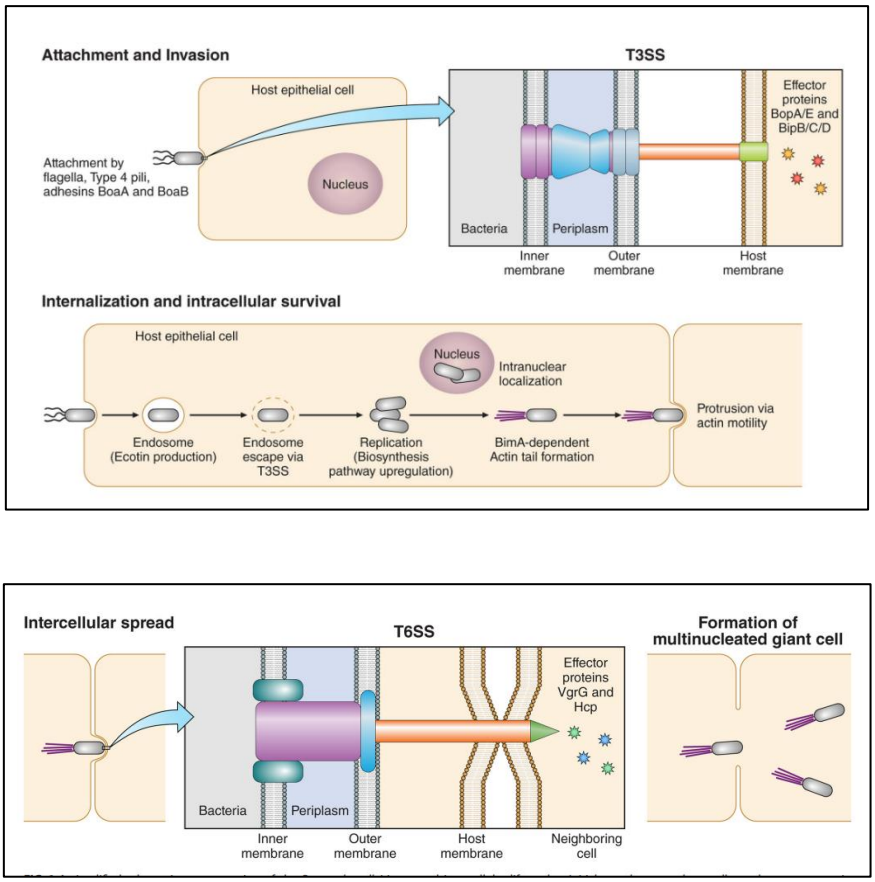

Figure 2.4 A schematic representation of the *B. pseudomallei* intra- and intercellular life cycles (Gassiep et al., 2020).

2.2.2 Type III Secretion Systems (T3SS) of *B. pseudomallei*

The versatility of *B. pseudomallei* as a pathogen is reflected in its huge 7.24 Mb genome organized into two chromosomes. One of the most important virulence factors that has been partially characterized in *B. pseudomallei* is its Type III Secretion Systems (T3SS). T3SS is documented as crucial virulence factors in numerous animal- and plant-pathogenic bacteria because it serves as a toxin delivery mechanism, enabling the pathogenic bacteria to inject toxic substance into the cytoplasm of the host cell. *B. pseudomallei* possesses three T3SS which are referred to asT3SS-1, T3SS-2, and T3SS-3. Each T3SS typically consists of a cluster of about 20 genes encoding structural components, chaperones and effectors which assemble into an apparatus resembling a molecular syringe that is inserted into the host cell membrane for the delivery of bacterial effectors into host cell cytosol (Lee et al., 2010). The genome of

B. pseudomallei consists of two circular chromosomes, with all three T3SSs 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 (Vander Broek and Stevens, 2017). Furthermore, the previous study reported that T3SS-1 gene cluster encompassing part of open reading frame 2 *(orf2)* and open reading frame 11 *(orf11)* were found to be present in *B. pseudomallei* and not in the related *B. mallei* or *B. thailandensis*. This finding indicates that *orf2* and *orf11* serve as specific markers for *B. pseudomallei* and were employed in this study (Chantratita et al., 2008; Novak et al., 2006; Thibault et al., 2004). This open reading frame was later found to be present in all of a large number of Northern Australian *B. pseudomallei* strain. In the case of T3SS-3, it is required for *B. pseudomallei* to efficiently escape the endocytic vesicle and is better characterized as homologous to the Inv/Mxi-Spa secretion systems of *Salmonella spp*. and *Shigella flexneri*, respectively.

2.3 Method for diagnosis melioidosis

Conventional culture method is the routine gold standard for diagnosis melioidosis, which is time-consuming and requires 5–7 days and the culture of *B. pseudomallei* is performed only in a highly equipped biosafety level 3 laboratory (Selvam et al., 2021). In addition, this method has a limited diagnostic sensitivity, this may be because of the low *B. pseudomallei* numbers in clinical samples or the presence of unculturable forms of the organism that have been associated with previous antibiotic treatment in some patients. There are many cases that have been under/misdiagnosed as a *Pseudomonas* species because of similar colony morphology in blood agar, Gram staining and biochemical tests such as positive oxidase test. The detection of *B. pseudomallei* is difficult in routine culture media because it mimics contaminants, and the overgrowth of normal flora is observed (Selvam et al., 2021).

Molecular methods such as PCR, real-time PCR and loop-mediated isothermal amplification (LAMP) assays have prevailed for diagnosis. However, these methods require an expensive instrument and professional operation, which limits their application in the field. Currently, recombinase polymerase amplification (RPA) is

developed to detect specific DNA of *B. pseudomallei* with high sensitivity and specificity (Peng et al., 2019).

A variety of serological tests have been developed in previous years, such as the enzyme-linked immunosorbent assay (ELISA) and the indirect hemagglutination assay (IHA), and a rapid bedside immunochromatographic test. These tests are challenging for diagnosis due to a lack of international standardization and high seropositivity rates in healthy individuals. This is because of high background seropositivity in areas where the disease is endemic, combined with delayed or absent seroconversion of some patients with melioidosis. The previously study in Thai population, an IHA cutoff titer of less than 1:80 was deemed unlikely to indicate a true positive, as 21% of healthy blood donors were found to have a titer of 1:40, titers of 1:80 to 1:320 were suggestive of infection, and a titer of 1:320 was very likely to indicate infection with a specificity of 97% (Gassiep et al., 2020). Although a monoclonal antibody-based latex agglutination test and direct immunofluorescent microscopy (DIF) have been developed for use with fresh clinical specimens and fast bacterial identification after laboratory culture, respectively, neither reagent is available commercially (Chantratita et al., 2008). An alternative technique for speedy and simple DNA amplification under isothermal conditions is loop-mediated isothermal amplification (LAMP), which just needs a heat block or laboratory water bath that keeps the temperature between 60 and 65 °C constant (Chantratita et al., 2008). The serodiagnosis of melioidosis remains a challenge but still has a role to play in the diagnosis of chronic melioidosis and where culture may not always be possible, such as in neuromelioidosis or with deep-seated abscesses. Perseverance in research and development may yield a fast, easy-to-use, and cost-efficient method specifically beneficial to resource-limited settings. Hence, it is not suitable for use in the field. Therefore, a simple, rapid, accurate as well as field-applicable diagnostic method for melioidosis is urgently needed.

2.4 CRISPR/CAS: CRISPR (Clustered regularly interspaced short palindromic repeats)

CRISPR (Clustered regularly interspaced short palindromic repeats): The CRISPR-Cas system evolved as a bacterial immune system to combat the invasion of phages and other mobile genetic elements like plasmids and transposons. There are three major steps involved in the evolution of CRISPR-Cas systems in bacteria. The first step is CRISPR adaptation that during this step, foreign invader genomic fragments are integrated into a CRISPR array as spacer sequences. Subsequently, crRNA Biogenesis, the CRISPR array is transcribed into pre-crRNA, which is then processed to form mature crRNA. Finally, CRISPR interference, during this final step, the crRNA effector complexes play a crucial role in defending against invading genetic elements. These crRNAs integrate with Cas effector proteins to create crRNA effector complexes. These programmed effector complexes identify and catalyze sequence-specific destruction of foreign invading genomic fragments.

CRISPR was first discovered in the 1980s and has become the tool of choice for genome editing. Currently, all identified CRISPR/Cas systems are classified into two main classes that are further subdivided into different types and subtypes based on the organization of their loci and signature proteins. Class I CRISPR/Cas systems include type I, III, and IV, which employ multi-subunit effector complexes. Conversely, Class II CRISPR/Cas systems use a single RNA-guided, multi-domain Cas proteins to recognize and cleave target sequences. Class II CRISPR/Cas systems encompass multiple types, including type II systems such as Cas9, type V, including subtypes Cas12 and Cas14 (designated now as Cas12f), and type VI, including Cas13 systems. After recognition of the target sequence guided by a single guide RNA, (sgRNA), a CRISPR-associated nuclease (Cas) cleaves the target DNA, creating a site-specific DNA double-strand break (DSB). The structure of the sgRNA scaffold depends on the Cas protein used. CRISPR/Cas9 is most widely used for genome engineering applications. However, different Cas enzymes have different activities that can be advantageous for diagnostic applications. CRISPR/Cas12a produces staggered-end DSBs, CRISPR/Cas13 targets single-stranded RNA (ssRNA), and CRISPR/Cas14 targets single-stranded DNA (ssDNA). Interestingly, following recognition and cleavage of the specific target, Cas12a, Cas13,

and Cas14 exhibit collateral, non-specific activities against ssDNA or ssRNA. These activities can be utilized for nucleic acid detection applications (Aman et al., 2020).

Recently, next-generation molecular diagnostics technology RNA-guided CRISPR/Cas nuclease-based nucleic acid detection has been developed and demonstrate high sensitivity, specificity and reliability. CRISPR/Cas technology has been successfully used for pathogenic nucleic acid detection such as viral infection; Zika virus , Dengue virus, human papillomavirus, Avian Influenza A Virus (H7N9) and SAR-CoV2 (Broughton et al., 2020; Wang et al., 2020). The detection relies on the target-activated nonspecific endonuclease activity of Cas13 or Cas12 after binding to a specific target RNA or DNA via programmable guide RNAs. By combining the programmable specificity of Cas12/13 with a reporter molecule that is activated upon target recognition, these enzymes result in specific and sensitive indications of the presence or quantity of nucleic acid (Ding et al., 2020; Wang et al., 2020).

2.4.1 CRISPR/Cas12a

Type V CRISPR/Cas system (comprised of subtypes V-A and V-B) is also known as Cpf1 (type V-A) or C2c1 (type V-B). The system was identified in organisms such as *Francisella novicida*, *Acidaminococcus* sp., *Lachnospiraceae* sp., *Prevotella* sp. which the enzyme is in size of ~1,100–1,300 amino acids. The total guide length for Cas12a is 42-44 nucleotides (nt) with the first 19-21 nt corresponding to the repeat sequence and the remaining 23-25 nt to the spacer sequence. The endonuclease produces a staggered cut on a PAM (region of 5-TTTV) distal site on the DNA with a 5 nt overhang on the target strand, and the PAM distal end of the cleaved product is then released from the complex.

Cas12 processes its own guide RNAs, leading to increased multiplexing ability. Cas12 has also been engineered as a platform for epigenome editing, and it was recently discovered that Cas12a can indiscriminately chop up ssDNA once activated by a target DNA molecule matching its spacer sequence. This property makes Cas12a a powerful tool for detecting the target DNA in a mixture. CRISPR/Cas12a (Cpf1) proteins are RNA-guided DNA targeting enzymes that bind and cut DNA as components of bacterial adaptive immune systems. It can be used as a powerful genome editing tool

based on its ability to induce genetic changes in cells at sites of double-stranded DNA (dsDNA) cuts. This target-activated non-specific ssDNase activity, catalyzed by the same active site responsible for site-specific dsDNA cutting, is also a fundamental property of other type V CRISPR-Cas12 enzymes. Activation of ssDNA cutting requires faithful recognition of a DNA target sequence matching the 20-nucleotide guide RNA sequence can distinguishing closely related DNA sequences. Following recognition of the target sequence, Cas12a exhibit collateral non-specific catalytic activities that can be employed for nucleic acid detection, for example by degradation of a labeled nucleic acid to produce a fluorescent signal (Aman et al., 2020).

Cas12a (Cpf1), derived from *Acidaminococcus sp.* recognizes the PAM sequence 5′ -TTTV-3′ (where V represents A, G, or C) the TTTV PAM site is on the strand opposite to the targeted strand which it employs only a CRISPR RNA (crRNA) or guided RNA (gRNA), which functions as the guide RNA, directing Cas12a to the target sequence. Unlike other systems, it does not rely on a trans-activating CRISPR RNA (Figure 5). Cas12a with a bound gRNA targeting a genomic site creates a double strand cut with staggered ends.

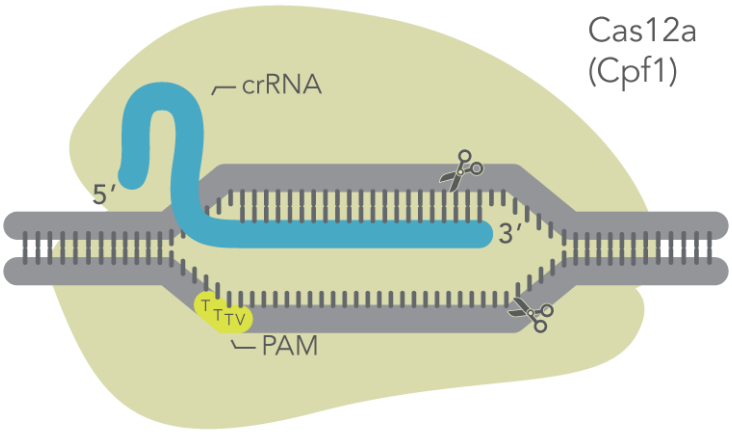

Figure 2.5 Cas12a (Cpf1) with a bound gRNA targeting a genomic site recognize the PAM sequence 5′ -TTTV-3′.

In previous study of detection of *B. pseudomallei* using CRISPR/Cas12a based on specific sequence tags demonstrated the specifically identify *B. pseudomallei* in less than 40 min. In addition, the dual-target RPA-CRISPR/Cas12a assay showed a high sensitivity as this assay can detect approximately 0.2 copies/reaction and 10 fg genomic DNA for LC1 and 2 copies/reaction and 20 fg genomic DNA for LC2 (Zhang et al., 2023). Recently, Wongpalee and cowoker reported the highly specific and sensitive of *B. pseudomallei* genomic DNA detection using CRISPR/Cas12a that crBP34-based detection assay could detect as few as 40 copies of *B. pseudomallei* genomic DNA while discriminating against other tested common pathogens. Moreover, genomic DNA of *B. pseudomallei* detection by CRISPR/Cas12a coupled with a lateral flow dipstick that the assay readout simply performed without the loss of sensitivity and does not require expensive equipment (Wongpalee et al., 2022).