IDENTIFICATION, CHARACTERIZATION AND PATHOGENICITY OF *AEROMONAS SCHUBERTII* ISOLATED FROM DISEASED STRIPED SNAKEHEAD FISH (*CHANNA STRIATA*)

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การพิสูจน์ การหาคุณสมบัติ และความสามารถในการก่อโรค ของเชื้อแอโรโมนาส ชูเบอร์ติไอ ที่แยกได้จาก ปลาช่อนนาป่วย

นายเฮนดรี บูดิแยนซา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	IDENTIFICATION, CHARACTERIZATION AND PATHOGENICITY OF *AEROMONAS SCHUBERTII* ISOLATED FROM DISEASED STRIPED SNAKEHEAD FISH (*CHANNA STRIATA*)
By	Mr. Hendri Budiyansah
Field of Study	Veterinary Science and technology
Thesis Advisor	Associate Professor CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P.

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-ปลาช่อนนา (*Channa striata*) เป็นหนึ่งในชนิดปลาที่นิยมเพาะเลี้ยงในประเทศไทย ปัญหาโรคติดเชื้อเป็นปัญหาที่มักหลีกเลี่ยงได้ยาก โดยเฉพาะอย่างยิ่งการติดเชื้อแคทีเรีย Aeromonas schubertii (A. schubertii) ในเดือนกรกฎาคม 2563 เกิดการระบาดของโรคในฟาร์มปลาช่อนในจังหวัดนครปฐม ประเทศไทย จากการทดสอบพบว่าเชื้อที่แยกได้คือ *A. schubertii* จำนวน 6 ไอโซเลท ซึ่งเป็นเชื้อก่อโรคหลักที่พบในปลาช่อนที่ฟาร์มแห่งนี้ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาคุณลักษณะและตรวจสอบการก่อโรคของ *A. schubertii* ที่แยกได้จากปลาช่อนนาป่วย การตรวจพิสูจน์เชื้อทำด้วย การวิเคราะห์ทางชีวเคมี PCR การหาลำดับดีเอ็นเอของยีน (16S rRNA และ *gyrB*) และ MALDI-TOF MS ผลการศึกษาพบว่า เชื้อ *A. schubertii* สามารถพิสูจน์แยกได้จากแบคทีเรียก่อโรคอื่น ๆ ด้วยลักษณะทางชีวเคมี DNA fingerprints (16S rRNA และ *gyrB*) และโปรตีนแมสสเปคตรัม จากการทดสอบการติดเชื้อโดยนำเชื้อ *A. schubertii* ฉีดเข้าในช่องท้อง (IP) ของปลาช่อนนา พบอัตราการตายในปริมาณที่สูงถึงร้อยละ 80 (1.2×10^8) และค่ากลางของระดับของเป็นพิษที่ทำให้ปลาตายร้อยละ 50 (LD_{50}) อยู่ที่ 3.9×10^5 รอยโรคสำคัญที่พบคือ แผลที่ผิวหนัง การตกเลือดในอวัยวะภายใน และตุ่มสีขาวที่อวัยวะภายในของปลา (ตับ ไต และม้าม) ซึ่งค่อย ๆ พบขึ้นในวันที่ 7 14 และ 21 วันหลังฉีดเชื้อ (dpi) โดยตุ่มสีขาวปรากฏขึ้นอย่างมีนัยสำคัญที่ตับ ม้าม และไต ตามลำดับ ในทำนองเดียวกันในการวิเคราะห์ทางจุลพยาธิวิทยา พบ granulomas, melano-macrophage centers (MMCs) และ necrotic cells นอกจากนี้ยังพบว่าตุ่มสีขาวที่มองเห็นทางมหภาพบนไตและม้ามมีความสัมพันธ์อย่างสูง (strongly correlated) กับ granulomas ที่ตรวจพบในเนื้อเยื่อไตและม้ามทางจุลพยาธิวิทยา

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Striped snakehead fish (*Channa striata*) is one of the popular cultivated fish in Thailand. However, it is inevitable to be susceptible to abundant pathogens, especially *Aeromonas* schubertii. In July 2020, an unknown outbreak was occurred on striped snakehead fish nursery farm in Nakhon Pathom province, Thailand. Suspected six isolates were *A. schubertii* yielded from the fish as the main causative agent. This research aimed to characterize and examine pathogenicity of *A. schubertii* isolated from diseased striped snakehead fish. Characterization was performed based on combination of biochemical assay, PCR and DNA sequencing (based on 16S rRNA and *gyrB*), and MALDI-TOF MS. The results showed the *A. schubertii* isolates can be distinguished from other bacterial pathogens by its biochemical characteristics, DNA fingerprints (16S rRNA and *gyrB*), and protein mass fingerprints. The experimental challenge of *A. schubertii*, using intra-peritoneal (IP) injection on *C. striata*, showed 80% mortality on high dose ($1.2x10^8$) and median lethal dose (LD_{50}) of $3.9x10^5$. A significant manifestation of skin ulceration, internal organ hemorrhage and white nodules on the fish internal organs (liver, kid-ney, and spleen) was progressively seen on 7-, 14-, and 21-days of post injection (dpi). The white nodules significantly appeared at the liver, spleen, and kidney, respectively. Likewise, granulomas, melano-macrophage centers (MMCs) and necrotic cells were also observed in histopathology analysis. Moreover, it was discovered that white nodules on kidney and spleen were strongly correlated with granulomas observed on kidney and spleen histopathology.

Field of Study:	Veterinary Science and technology	Student's Signature .....
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CHAPTER I IMPORTANT AND RATIONALE

The striped snakehead fish (*Channa striata*) becomes more well-liked aquaculture among worldwide countries. It has been reported as one of the preferred cultivated species In world aquaculture and approached 1% of total world finned-fish aquaculture production beneath tilapia (4%) and catfish (2%) in 2016 (FAO, 2018), 2018). Moreover, Thailand is one of the successful countries on freshwater aquaculture industry, including snakehead fish as one of the biggest cultivated freshwater fish species (Pongsri and Sukumasavin, 2005; FAO, 2018). Thailand's snakehead fish farm has been rapidly rising since 1970 and it reached 7,255.56 tons of production valued at 11,367,258 USD in 1981 and made up to 14,444 tons in 2003 valued at 112 million USD in 2003, it reached almost 2.44% out of Thailand's freshwater aquaculture production (Boonyaratpalin et al., 1985; Piumsombun et al., 2005; Pongsri and Sukumasavin, 2005). In addition, snakehead fish reached 15.4% of total fish consumption in Thailand beneath tilapia (29%) and silver barb (16.3%) (FAO, 2009). However, snakehead fish is able to be susceptible to various pathogens such as parasites, viruses, fungi and bacteria (Courtenay and Williams, 2004). Snakehead fish are susceptible to bacterial diseases which are commonly caused by *Aeromonas sp.* (Duc et al., 2013), *Acinetobacter baumannii* (Rauta et al., 2011), *Pseudomonas fluorescens* (Qureshi et al., 1999), *Mycobacterium sp.* (Densmore et al., 2016) and *Norcadia* sp. (Wang et al., 2007). The *Aeromonas*

schubertii, a gram-negative short rod-shaped bacterium and a member of Aeromonadaceae family, has emerged as a main problem in cultured snakehead (Chen et al., 2012; Liu and Li, 2012) and brackish water Nile tilapia (Ren et al., 2019) in China recently. The fish had been reported showing typical *Aeromonas* symptoms such as lethargy, listless swimming, mouth and fin hemorrhages and abdominal distension (Chen et al., 2012; Liu and Li, 2012). Liu and Li (2012)'s study showed the fish manifested with multiple ivory white nodules scattered throughout the fish kidney with a diameter of 0.5 – 1 mm. moreover, it caused 45% mortality of 18-month-old snakehead fish (*Ophiocephalus argus*) within 40 days. Furthermore, several studies had been reported this novel bacterium disease is on world-wide aquaculture industry such as rainbow trout (*Oncorrhyncus mykiss*) in Turkey (Akaylı et al., 2011), doctor fish (*Garra rufa*) (Yu et al., 2009) in China, mussels and oysters in Spain (Latif-Eugenín, 2016) and shrimp (*Peneaus vannamei*) in Thailand (Sangpo et al., 2020).

In Thailand, *A. schubertii* has been recorded in striped snakehead farms in Nakhon Pathom Province since 2020 (our data). The diseased fish showed clinical signs of mouth, basal fin and internal organs hemorrhages, and multiple white spot nodules in internal organs (spleen, kidney). Initial identification based on combined method by biochemical test and MALDI-TOF MS assay showed that the pre-dominated bacteria isolated were identified as *A. schubertii*. Unfortunately, there is no report or record of *A. schubertii* causing disease in cultured striped snakehead fish

in Thailand. Therefore, this study aims to characterize and examine pathogenicity of *A. schubertii* in cultured striped snakehead (*Channa striata*).

CHAPTER II LITERATURE REVIEW

2.1 Striped snakehead (*Channa striata*) aquaculture.

The striped snakehead or Asian snakehead fish belongs to *Channidae* family natively found in drainages, rivers, and canals of South and Southeast Asia countries such as India, Bangladesh, Nepal, Cambodia, Myanmar, Indonesia, and Thailand. Moreover, it was introduced to Hawaii in early 1900 and to the USA in early 1990 (Nelson, 1994; Courtenay and Williams, 2004). Thailand has been cultivating the striped snakehead fish using the monoculture system in Suphan Buri, Nakhon Pathom, Samut Sakhon, Samut Songkhram, Nakhon Nayok, Samut Prakan, Chanthaburi, and Trat provinces since 1970 (Boonyaratpalin et al., 1985). Striped snakehead fish farm in Thailand reached almost 7,255.56 tons of production valued at 11,367,258 USD in 1981 and almost 20,000 tons in 2003 valued at 0.1 billion USD in 2003 (Pongsri and Sukumasavin, 2005). It was accounted for 0.9% of total Thailand's freshwater aquaculture productions (Figure 1) which is 381,000 tons of total production in 2016 (DoF, 2018).

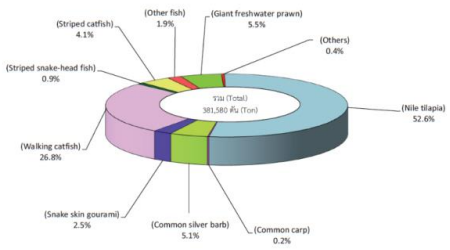

Figure 1. The major freshwater aquaculture species production in Thailand in 2016 (DoF, 2018).

The striped snakehead fish (*C. striata*) prefers to live in freshwater ponds and streams and it tolerates in quite foul and muddy water in which has a pH value at 4.0 - 5.0 (desirable pH value 6.5 - 8.0) in tropical temperature (28 ^{o}C) (Lee and Ng, 1991). *C. striata* is able to survive in the low dissolved oxygen conditions because it has air-breather ability from late juvenile stages (Courtenay and Williams, 2004). Qin et al. (1997) had reported that it was able to survive in below 50 mg of total ammonia-nitrate (TAN) per liter for 96 hr exposure. The female snakehead fish begin to be mature about 30 cm in length at two years of age and starts mating in rainfall season. More than a thousand amber-colored eggs are able to be laid by single brood stock which it will hatched within 1 or 3 days (Lee and Ng, 1991; Talwar and Jhingran, 1991). An adult striped snakehead fish is a carnivore which feeding live animals such as small fishes, prawns, frogs, and invertebrates. Moreover, a small fry mainly feeds zooplankton until it passes the fry stage within ten days. In the fingerling stage, it turns into orange color and becomes more independent. Insects, water fleas and other fish fry are preferable food for *C. striata* fingerling until it reaches an average weight of 60 g in 12 weeks as a juvenile (Jhingran, 1984; Courtenay and Williams, 2004).

2.2 Motile *Aeromonas* Septicemia (MAS) in fish

The aeromonad family is considered as a ubiquitous pathogen in the aquatic environment. Surface and underground waters as well as residual and irrigation

waters have been reported that have high incidences of *Aeromonas* (Janda and Abbott, 2010; Salvat and Ashbolt, 2019). Not only it is able to be found in the aquatic environment, it can also be discovered in daily food product such as drinking water (van der Kooij et al., 2015), fruits and vegetables (Latif-Eugenín et al., 2017); dairy product (Freitas et al., 1993); meats (Chang et al., 2008; Nagar et al., 2011; Praveen et al., 2016); fresh fish, fish eggs and shrimps (Hänninen et al., 1997); and shellfish (Abeyta Jr et al., 1990). Moreover, Aeromonadaceae has been reported that it was recovered from dung of rabbits, dogs, cats and horses (Ghenghesh et al., 1999; Forga-Martel et al., 2000). The *Aeromonas* genera also had been reported that it was associated with gastroenteritis (as they have cytolytic enterotoxin), septicemia and wound infection in human (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015).

In animals, this *Aeromonas* genus has been reported causing ulceration and hemorrhages in frogs (Pearson et al., 2000). Other reports discovered it was as the pathogen in echinoderms, mollusks, crocodile, and copepods (Minana-Galbis et al., 2007; Gugliandolo et al., 2008; Pu et al., 2019). The *Aeromonas* is well-known wide-worldly as a severe pathogen in aquaculture called motile *Aeromonas* septicemia (MAS) (Beaz-Hidalgo and Figueras, 2013). The noticeable *Aeromonas* species which causing motile *Aeromonas* septicemia for fish aquaculture are summarized in table 1. On the other side, Hossain et al. (2014) and Rasmussen-Ivey et al. (2016) have reported that hypervirulent *Aeromonas hydrophila* (vAh) had been infected farmed

channel catfish in Mississippi River, Alabama, USA in 2009. This strain caused the rapid onset of mortality and wide range severity of hemorrhages in fish fins, head, and internal organs. It also had been reported causing atypical symptoms in Atlantic cod (Magnadóttir et al., 2002). Another *Aeromonas* which were responsible of septicemia were *Aeromonas veronii* biovar (bv) *veronii* and *Aeromonas jandaei*. Other symptoms caused by these bacteria were fin rot, exophthalmia, cloudy eyes and severe hemorrhage in the liver and brain in Nile tilapia (Dong et al., 2017).

Table 1. Well-known causative agents of *Aeromonas* disease in fish.

Species	Disease and Host	Location	References
Aeromonas hydrophila	MAS in Arapaima gigas	Brazil	Dias et al. (2016)
Aeromonas veronii bv. *veroniii*	infection in catfish	USA	Nawaz et al. (2006)
Aeromonas veronii bv. *sobria*	infection in catfish, carp and snakehead	Bangladesh	Rahman et al. (2001)
Aeromonas piscicola	infection in salmon and trout (2005)	Spain	Beaz-Hidalgo et al. (2009)
Aeromonas bestiarum	Infection and fin rot in ornamental goldfish	UK	Robertson et al. (2005)
Aeromonas allo saccharophila	infection in rainbow trout	India	Shahi et al. (2014)
Aeromonas caviae	infection in Indian catfish	India	Thomas et al. (2013)
Aeromonas dhakensis	infection in ornamental fish (2005)	Portugal	Martínez-Murcia et al. (2008)
Aeromonas schubertii	infection and white spot nodules in northern snakehead fish (2009)	China	Liu and Li (2012)
Aeromonas jandaei	infection in European eels	Spain	Esteve et al. (1995)

Most of *Aeromonas* species possesses a single polar and lateral flagella to help them to move "swarming and swimming" in a solid or liquid medium (Fernández-Bravo and Figueras, 2020). Another *Aeromonas* morphology is its bacterial pili which can be used as the sexual purpose, secretion system and secreted substance (Cytotoxic enterotoxin, hemolysin, lipase, protease, amylase, etc.) as their virulence factor (Figueras and Beaz-Hidalgo, 2015; Fernández-Bravo and Figueras, 2020). Recent reports had been reported that its bacterial capsules, Lipopolysaccharides (LPS), S layer, biofilm information and quorum sensing may also help the bacteria may involve in different mechanisms against host immune responses and invade the host. These *Aeromonas* virulence features may contribute the bacterium on host cell adhesion, invasion and evasion from host immunity systems (Fernández-Bravo and Figueras, 2020). These features also may wind-up the route of infection as in the water or food contamination, wound penetration and blood-borne in the human model as well as in animal model (Liu, 2015; Fernández-Bravo and Figueras, 2020). Experimental infection studies in fish reported the *Aeromonas sp.* is mostly able to invade the host by route of infection of gills (Zepeda-Velázquez et al., 2017; Ran et al., 2018), skin and muscular penetration (Huizinga et al., 1979; Rahman et al., 2001), gastrointestinal or per-oral (Markwardt and Klontz, 1989; Lio-Po et al., 1998), intraperitoneal (Magnadóttir et al., 2002; Ren et al., 2020), egg exposure (Bergh et al., 1997) . it was as well reported that *Aeromonas sp.* is able to infect fish through vertical transmission (Cipriano and Bullock, 2001).

Stress conditions, such as fluctuated temperature (Rahman et al., 2001), high nitrite concentration (Chand and Sahoo, 2006), low dissolved oxygen (DO) (Shen et al., 2010) and various pH conditions (Whipple and Rohovec, 1994) in water may also affect the virulence of *Aeromonas* infection and host immune systems.

2.3 *Aeromonas schubertii* disease in fish.

The *A. schubertii* as a member of the *Aeromonas* genus is commonly inhabited in the aquatic environments. The bacteria had been reported it was able to be recovered from different aquatic habitats and animals, such as frog (Pearson et al., 2000), mussels (Latif-Eugenín, 2016), ornamental fish (Smith et al., 2012), rainbow trout (Akaylı et al., 2011), shrimps (Yano et al., 2015; Sangpo et al., 2020). Moreover, food products had been reported it had high incidences of *A. schubertii,* such as rabbit meat and chicken meat (Rather et al., 2014), pasteurized milk and cheese (Freitas et al., 1993), and drinking water (Razzolini et al., 2008). Recently, new emerged pathogen had been reported that *A. schubertii* was a main problem of cultured snakehead in China. This bacterium had ability causing 45% mortality on adult northern snakehead fish (*Ophiocephalus argus*) within 40 days (Liu and Li, 2012) and high mortality within 2 weeks in blotched snakehead fish (*Channa maculata*) (Chen et al., 2012). Infected fish are often manifested hemorrhages on the skin, around the mouth and multiple ivory white nodules with a diameter of 0.5 - 1 mm in the kidney. Internal organ (spleen, liver, kidney) tissues of the *A. schubertii*

infected snakehead fish showed multifocal granulomatous lesions, with the appearance of numerous small, cocco-bacilli bacteria. Severe congestion of hepatoportal blood vessels and vacuolar degeneration, depletion of hematopoietic cells and vascular thrombosis, vacuolation and interstitial hemorrhage were observed in liver, spleen and kidney of *A. schubertii* infection (Liu and Li, 2012). Nile tilapia and zebrafish had been reported they were manifested with necrosis, congestion, with- or without- white necrotic foci in liver and kidney as the main symptoms of *A. schubertii* infection (Liu and Li, 2012; Liu et al., 2018; Ren et al., 2019). *A. schubertii* had been also reported it was strongly virulent to snakehead and Nile tilapia with the lethal doses (LD_{50}) of 1.4×10^4 and 4.91×10^4 CFU/g fish by intraperitoneal (ip) injection, respectively (Chen et al., 2012; Ren et al., 2019).

2.4 Host-Pathogen Interaction of *A. schubertii*.

Vertebrates possess complex immunity systems to defend themselves against the pathogens. As in other vertebrates, fishes have its own complex innate, humoral, and specific adaptive immunity as their protection against pathogenic microorganisms inhabited in their natural habitat. The innate immunity has major important role in fish against bacterial infection because adaptive immune system in fish is less developed than those in higher vertebrates (Plouffe et al., 2005). Based on its morphology, The fish immunity is represented as cellular and molecular components. Cellular components are composed of various types of leukocytes

such as neutrophils, basophils, eosinophils, macrophages, non-specific cytotoxic cells (NCC), natural killer-like cells (NK-like cells) as well as lymphocytes T and B. Also, The molecular components are consisted of mucosal or epithelial barrier in skin, gill and alimentary tracts, lectins, complement proteins, antimicrobial peptides (AMP), cytokines, the receptors such as pattern recognition receptors (PRRs) and the novel immune-type receptors (NITRs) (Plouffe et al., 2005; Aoki, 2008). Immunoglobulins (Ig) are also able to be found in teleost immunity systems. For example, the IgM is commonly presented in fish, yet the IgD and the IgT/Z are less presented (Uribe, 2011).

The infection starts with the pathogens (bacteria, viruses, and fungi) attachment to the skin surfaces. Then, viscous mucus is produced by epithelial cells in host skin with its antimicrobial peptides, lectin, complements, IgM and cytokines starts to prevent the pathogens to entering the skin (Uribe, 2011). When the pathogen start to enter the skin or tissue, the leucocytes initiate maturation, migration, preparation of pathogen-associated molecule pattern (PAMPs) recognition by using PRRs, phagocytosis, presenting antigen on cell surface, releasing cytokines and inducing inflammation (Aoki, 2008). The lymphocyte T is matured as cytotoxic T cell, T-helper 1 and T-helper 2. Then, it starts to release the cytokines prior to induce lymphocytes B maturation. The lymphocytes B releases immunoglobulin to neutralize the pathogens after its maturation has been done (Aoki, 2008; Uribe, 2011).

Kokka et al. (1992) had reported that *A. schubertii* is possessed a LPS substance which resemble to *A. hydrophila* and *A. veronii*. This microbial product may cause unspecific immune response of the host such as TLR 4 which Is a PRR on macrophage's surface cell (Aoki, 2008). In addition, Ren et al. (2020) also had reported that the elevated expression of IL-1β, TNF-α, TGF- β and HSP-70 in head kidney and liver of *A. schubertii* experimental infection on Nile tilapia was caused by bacterial LPS invasion throughout the tissue. In short, these elevations may cause inflammatory cascade such as leucocytes invasion, phagocytosis activities and immunoglobulin secretion occurred in the target area (Aoki, 2008).

Recent study had reported accumulation of melano-macrophage centers (MMCs) had been found on acute *Aeromonas* infection in Nile tilapia's internal organ (liver, spleen, kidney and brain) (Fernandes et al., 2019). The MMCs is consider as non-immunity response which it engulfs cell debris, such as dead erythrocytes or immune system waste products which is a byproduct of interaction between host defense systems with the pathogens prior of detoxification, destruction and recycling of microbial toxins or products (Agius and Roberts, 2003). The MMCs is also possible to be indicator of antigen retention and resistant intracellular organism due to its association with lymphoid cells and highly production of antibody (Agius and Roberts, 2003). Moreover, The *Aeromonas schubertii* has been reported it possesses with toxin called the cytolytic enterotoxin or beta-hemolysin which are associated

with those of which *A. hydrophila* and *A. veronii* recovered from gastroenteritis patients. This microbial product is mainly produced by T2SS secretion system of *Aeromonas* family (Kokka et al., 1992; Fernández-Bravo and Figueras, 2020).

The *Aeromonas schubertii* nodular symptoms are commonly mistaken as *Mycobacterium* sp., *Nocardia* sp, or *Francisella* sp. infection (Liu and Li, 2012). The white ivory nodules in fish's internal organ are often resembled as granulomatous lesion which is formed by accumulation of immune cells (infected and non-infected macrophage, epithelial cell and fibroblast) into heterogeneous compacted foreign mass (Huang et al., 2021; Martínez-Lara et al., 2021). The compacted mass is stimulated by pro- and anti- inflammatory agents from phagocytosis failure or persisting pathogens as well as its toxins (Martínez-Lara et al., 2021). The T3SS and T6SS of *Francisella* sp. and *Nocardia* sp. had been reported it plays important role as internalization, replication, and survivability of intracellular organism, such as by suppressing expression of IL-1β and IL-8 but upregulated the IL-10 and IL-12/17 which is the anti-inflammatory cytokines inside infected host cell or macrophage (Munang'andu, 2018). There is no available in-depth study concerning pathogenesis and host defense against *Aeromonas schubertii* infections in snakehead fish.

2.5 Taxonomy and characterization of *A. schubertii*.

The *Aeromonas schubertii*, according to Hickman-Brenner et al. (1988), was firstly found in human specimens, such as blood, abscesses, wound, skin and pleural fluid as vernacular. Later, it was named enteric 501. it is classified as follows:

Phylum : Proteobacteria

Class : Gammaproteobacteria

Order : Aeromonadales

Family : Aeromonadaceae

Genus : *Aeromonas*

Species : *A. schubertii*

The *A. schubertii* is commonly a short-rod gram negative bacteria and has typical characteristics, such as positive reaction on methyl red (MR), voges-proskauer (VP), arginine and lysine decarboxylase, oxidase, motility, and lipase assay. However, it shows negative reaction in esculin hydrolysis and mannitol fermentation assay (Hickman-Brenner et al., 1988). Abbott et al. (2003) had reported that *A. schubertii* is able to be distinguished within other *A. sobria* complex (*A. veronii*, *A. jandaei*, *A. schubertii* and *A. sobria*) by its phenotypic characteristics in indole, Voges-Proskauer test, lysine decarboxylase, L-arabinose fermentation, and lipase test. The phenotypic or biochemical characteristics of *A. schubertii* compared with other *Aeromonas* species can be seen in table 2.2. The *A. schubertii* had been discovered it has beta

hemolysin activity which is non-cell associated and it is not released into extracellular, unlike those of *A. hydrophila* and *A. veronii*. Moreover, a novel cytotoxin activity against Hep-2 cells has been reported which it is possessed to *A. schubertii* (Kokka et al., 1992). This species, similar to its family, is resistant to 0/129 vibriostat and able to inhabit in 0 - 3 % salinity of aquatic environment which it can be distinguish with *Vibrio sp.* (Carnahan et al., 1991).

Table 2. Phenotypic characterization of *A. schubertii* and other *Aeromonas* species

Characteristics	*Aeromonas schubertii* strains				Other *Aeromonas* species		
	HYL1 [a]	ZS20100725 [b]	ASS4 [c]	ATCC 43700 [d]	*A. hydrophila* [e]	*A. veronii* [f]	*A. salmonicida* [g]
Gram stain	- SR	- SR	- SR	- R	- SR	- R	- SR
Haemolysin	ND	ND	ND	+	ND	ND	-
Catalase	+	+	ND	ND	+	ND	+
Oxidase	+	+	ND	+	+	ND	+
Motility	+	+	+	+	+	+	-
O/F	F	F	ND	F	F	F	F
Indole	-	-	ND	-	V	+	+
Methyl red	+	+	ND	+	ND	+	ND
Voges Proskauer	-	V	ND	-	V	+	+
Lysine decarboxylase	+	-	+	+	+	+	-
Ornithine decarboxylase	-	V	-	-	+	-	-
Arginine Deaminase	+	-	-	+	+	+	+
Esculin	-	-	+	-	ND	ND	-
Gelatin	+	ND	+	+	+	+	+

Characteristics	*Aeromonas schubertii* strains				Other *Aeromonas* species		
	HYL1 [a]	ZS20100725 [b]	ASS4 [c]	ATCC 43700 [d]	*A. hydrophila* [e]	*A. veronii* [f]	*A. salmonicida* [g]
H_2S	-	-	-	-	-	-	+
Lactose	-	-	-	-	+	+	ND
Maltose	+	+	+	+	ND	ND	ND
Sucrose	-	-	-	-	V	+	+
D-Mannitol	-	-	-	-	V	+	+
D-Sorbitol	-	-	+	-	V	-	ND
L-Arabinose	-	-	-	-	V	-	ND

+ = positive result, - = negative result, ND = no data available; V = vary.

[a] Liu and Li (2012), [b] Chen et al. (2012), [c] Ren et al. (2019), [d] Hickman-Brenner et al. (1988); Carnahan et al. (1989), [e] Matter et al. (2018), [f] Dong et al. (2017), [g] Magnadóttir et al. (2002).

The commercial identification systems have been tested to identify the *Aeromonas spp.* such as Vitek-2 compact system (O'Hara and Miller, 2003; Funke and Funke-Kissling, 2004), API-20E (Santos et al., 1993; Awan et al., 2005), phoenix 100 ID systems (O'Hara, 2006), API-32GN (Awan et al., 2005), MicroScan walkaway (Vivas et al., 2000; Soler et al., 2003), and Omnilog GN2 microplate (Lamy et al., 2011; Prediger et al., 2017). They reported that these identification systems able to identify 77-98% into genus level of *Aeromonas* spp.. moreover, it was able to identify into species level such as *A. hydrophila, A. veronii and A. caviae* with accuracy 90-100%, 96.7-100%, 70-100% and 76-90% by using API 20E, API 32GN, Vitek2 GN and MicroScan W/A, respectively (Lamy et al., 2011). In contrary, it had been reported it was unable

to identify other *Aeromonas* species i.e. *A. jandaei*, *A. allosaccharophila*, *A. salmonicida*, and *A. schubertii* (Lamy et al., 2011). The confusion identification on *Aeromonas* spp. and *Vibrio spp.* was one of the obstacles of utilizing these identification systems. Due to its unsuccessful discriminations on *Aeromonas* spp. and *Vibrio* spp., incomplete logarithm was occurred based on dependent of a single characteristic such as antimicrobial susceptibility, acid production in sugars, and salt toleration, in view of vast variability of *Aeromonas* spp. characteristics (Abbott et al., 2003; Soler et al., 2003; Lamy et al., 2011).

In the genotyping of *Aeromonas* genus, the 16S rRNA gene allows distinct identification and comparison between *Aeromonas* species and other bacterial pathogen species, since it is distributed commonly in bacteria chromosomal genome (Weisburg et al., 1991; Demarta et al., 1999; Janda and Abbott, 2007). However, several cases had been reported 16S rRNA is unable to separate close-related species, such as *A. veronii* with *A. ichthiosmia* (Martinez-Murcia et al., 1992; Demarta et al., 1999; Janda and Abbott, 2007). Because this drawbacks on identification of *Aeromonas* spp. with this gene sequence, it needs combination method of identification with other genes to identify the *Aeromonas* spp., such as housekeeping genes (HKGs) (Fernández-Bravo and Figueras, 2020). The study of *Aeromonas* identification by utilization of housekeeping gene i.e. *gyrB* gene that encodes the subunit B or DNA gyrase showed smaller error of identification on inter-species (less than 3%) and intra-species (less than 2%) on *Aeromonas* genus (Yanez et al., 2003).

Another housekeeping gene study for *Aeromonas* was *rpoD* gene that encodes sigma factor S70 (Soler et al., 2004). Concatenated housekeeping genes have been used (i.e. *gyrA, dnaX, dnaJ, recD* and *atpD*) to describe the MLPA of *Aeromonas* genus showing huge distinct taxonomy classification (Martinez-Murcia et al., 2011).

2.6 Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption/ionization time of flight mass spectrometry or MALDI-TOF MS recent year has been introduced to clinical laboratories for identification of various species of Gram-negative and -positive. Moreover, it has been widely and commonly used in national and international institutions, such as the USA FDA (Schubert and Kostrzewa, 2017). The MALDI-TOF MS is able to describe the microorganism by matching the ionized sample protein spectra with the database spectra. MALDI-TOF MS ionize the sample protein by using laser with matrix assisting. Then, it flies through the vacuum chamber to the detector, and it is visualized in monitor recording as a spectrum (Glish and Vachet, 2003; Mellon, 2003).

At present, there were several studies agreed that MALDI-TOF MS is an accurate tool for identification of *Aeromonas* species levels. In particular, the identification accuracy of this tool was 100% for species and 97% for genus compared with the results of *rpoB* sequencing of 217 isolates (Chen et al., 2014). Shin et al. (2015) had re-identified 65 *Aeromonas* spp. using MALDI-TOF MS which

was previously identified by *gyrB* sequencing and reported the accuracy of genus level and species level at 98.5% and 92.3%, respectively. Benagli et al. (2012) had found 93% corrected identification in species level when re-identified 741 *Aeromonas* spp. strains from the environment by using MALDI-TOF MS and SuperSpectra™ database. Pérez-Sancho et al. (2018) had claimed the increasing number of corrected identifications of *Aeromonas* spp. strains isolated from fish after newly entries of 14 *Aeromonas* spp. spectra in the MALDI-TOF Biotyper™ database.

The MALDI-TOF MS assay had been reported which is able to identify 82% correctly of 41 *Aeromonas* strains including *A. schubertii* spectra (Pérez-Sancho et al., 2018). It is possible to distinguish of each species by using MALDI-TOF MS due to *Aeromonas* family protein complexity which yielding various spectra of each species (Schubert and Kostrzewa, 2017; Pérez-Sancho et al., 2018). The main limitation of MALDI-TOF is requirement of updated database in-house or even online including missing and newly additions of *Aeromonas* species spectra (Pérez-Sancho et al., 2018)

CHAPTER III MATERIAL AND METHOD

3.1 Sample collection and bacteria identification.

An unknown disease occurred at a striped snakehead fish farm in Nakhon Pathom province, Thailand in July 2020. Fifteen striped snakehead fish fingerlings were transferred to Fish Infectious Diseases Research Unit (FID-RU), Chulalongkorn University for diagnosis. The fingerlings showed erratic swimming and lethargy. Typical clinical signs of aeromonads infection were observed by external examination such as fin and tail hemorrhage and skin erosion. Necropsy and internal organ inspections had been conducted and showed internal organ hemorrhages. Moreover, visible white nodules in spleen, kidney, and hemorrhaged liver were also observed in internal examination. The accumulated mortality was approximately 40% within one month. It was noted that there was no ectoparasites and endoparasites observed by wet mount method. Moreover, there was no virus detected by using PCR assay. The pre-dominant colony isolated from spleen, kidney, and liver of the diseased fish was recovered and was undergone to initial biochemical assay and MALDI-TOF MS (Bruker Daltonics, Massachusetts, USA). The six isolates were renamed as CUVETASC03, CUVETASC04, CUVETASC05, CUVETASC06, CUVETASC07, and CUVETASC08. the details of six isolates can be seen in the appendix of this thesis. All isolates were preserved in tryptic soy broth (TSB) added 20% glycerol and kept at -80^{o}C for further work.

3.2 The Characterization of *A. schubertii*

The suspected *A. schubertii* isolates were recovered from glycerol stock at -80°C on tryptic soy agar (TSA) and incubated under 28°C for 24 h. After that, the pure bacteria colonies were subjected to identify by biochemical characterization by biochemical tests, molecular characterization by utilization of PCR with 16rRNA and *gyrB* primers, and MALDI-TOF MS assay.

3.2.1 Biochemical profile characterization of *A. schubertii* by using biochemical assay

The suspected *A. schubertii* isolates were grown on TSA at 28°C overnight. The pure colony were subjected to obtain biochemical characteristic profile by utilization of the conventional biochemical assays including gram staining, hemolysin test, oxidase test, catalase test, motility test, gelatin hydrolysis, lipid hydrolysis, casein hydrolysis, lecithinase test, oxidative-fermentative test, esculin hydrolysis test, indole test, Voges Proskauer (VP) test, methyl-red test, amino decarboxylation (arginine, lysine and ornithine), sugar fermentation test (mannitol, dextrose, arabinose, sucrose and lactose), gluconate test, nitrate reduction test and 0.5%-5.0% salt supplemented TSA (BD Difco™, New Jersey, USA). The isolates were cultured on each of the biochemical assay and incubated at 28°C for 24 hr. The biochemical characterization of *A. schubertii* was performed as previous publications (Abbott et al., 2003; Barrow and Feltham, 2004). the details of each biochemical assay preparation were described in the Appendix of this thesis.

Vitek-2 compact® GN card (BioMérieux, Marcy-l'Étoile, France) was used to obtain additional biochemical profile characterization of the six isolates of *A. schubertii* in this study. A pure single colony of *A. schubertii* isolates was collected and suspended on normal saline water (NaCl 0,95%). Then it was standardized by using MacFarland standard 0.5 with densitometer 600nm. The standardized bacterial suspensions were transfer into Vitek-2 compact® isolate tray connected with GN card in each isolate. The machine was set up to 35°C and the isolates were incubated for 24hr to obtain the results. The results of Vitek-2 compact® were combined with conventional biochemical assays and compared with biochemical profile of published *A. schubertii* data.

3.2.2 Characterization of *A. schubertii* by using 16S rRNA and *gyrB* gene.

The DNA of suspected *A. schubertii* isolates were extracted using the Queipo-Ortuño et al. (2008)'s boiling method. The method is described as followed: the bacterium is prepared as pellet by centrifuged bacterial broth solution at 15000 xg for 15 min. Then, it was resuspended with molecular biology grade water. After that, it will be subjected to boil in 100°C water bath for 10 min. Moreover, it is cooled on ice immediately for 15 min. Finally, it is centrifuged at 15000 xg for 10 sec and stored the supernatant in new tube at -20°C.

The extracted DNA was subjected to undergo polymerase chain reaction (PCR) assay using 16s rRNA and *gyrB* gene primers (table 3). Each reaction was carried

out in total volume of 25 µl containing 12.5 µl of Master Mix Go-Taq® Green (Promega, Madison, USA), 1 µl of DNA template (150-200 ng), 1 µl each of 10 pmol 9.5 µl ultra distilled water. The PCR condition was purposed for 16s rRNA primer was 25 to 35 cycles of 95°C for 2 min, 42°C for 30 s, and 72°C for 4 min, plus one additional cycle with a final 20-min chain elongation. And then, the PCR condition was purposed for *gyrB* gene primer was 35 cycles as follows: denaturation at 94°C for 30 sec, annealing 55°C for 30 sec and extension at 72°C for 1 min. The DNA amplicons were visualized on a 1-2% TBE-agarose gel containing 0.05 RedSafe™ dye (iNtRON, Seognam-si, South Korea). The electrophoresis running condition was 100 V, 400 mA for 30 min. The agarose gel was observed under an ultraviolet light.

Table 3. List of Primers and Primer sequences which was used in this study.

Primers	Sequence 5'-3'	References
16S rRNA gene	27F : AGAGTTTGATCATGGCTCAG	Weisburg et al. (1991)
	1492R : TACGGTTACCTTGTTACGACTT	
gyrB gene	3F : TCCGGCGGTCTGCACGGCGT	Yanez et al. (2003)
	14R : TTGTCCGGGTTGTACTCGTC	

In addition, the DNA amplicons were purified from agarose gel by using Nucleospin® Gel and PCR clean-up (Macherey-Nagel, Duren, Germany). Then, it was subjected for DNA sequencing by using Sanger sequencing. Finally, the forward and reverse sequences were assembled by using BioEdit® version 7.1.1 and analyzed by using NCBI-BLAST® network services with identity ≥ 99.5% for 16s rRNA and ≥ 99.0% for *gyrB* sequences (Janda and Abbott, 2007; Beaz-Hidalgo et al., 2015; Hoel et al.,

2017). The DNA sequences were analyzed by using MegaX® software to analyze the phylogenetic tree of the relativeness of *A. schubertii* strains with other bacteria. Details of *A. schubertii*, *Aeromonas* sp. and other bacteria from NCBI GenBank® as out group on the maximum likelihood phylogenetic tree are explained in the Appendix of this thesis.

3.2.3 Characterization of *A. schubertii* by MALDI-TOF MS assay

The *A. schubertii* isolates were undergone by extraction of biopolymers of the bacteria and measured by using a Bruker Daltonics UltrafleXtreme MALDI-TOF MS equipment. The isolates were recovered from glycerol stock on TSA and incubated at 27°C for 24 h. A 5g of colony of each isolate was taken and placed inside a centrifuge tube filled with 300 µl of high pressured liquid chromatrography (HPLC) grade distilled water, added 900 µl ethyl alcohol in the tube then centrifuged in 13000 x g for 2 min. removed the supernatant and centrifuged one more time, then, let it dry. Each of tube was added 30 µl of formic acid and 30 µl of acetonitrile and mixed briefly with vortex. Then, it was centrifuged in 13000 xg for 2 min. 1 µl of supernatant of each isolates was transferred to clean metal plate and mixed with CHCA (α-cyano 4-hydrocinnamic acid). Then, let it dry for few seconds. All samples were analyzed by a Bruker Daltonics UltrafleXtreme MALDI-TOF MS equipment and the FlexControl software v3.0 (Bruker, Massachusetts, USA) within mass range 2 to 20 kDa for acquisition mass spectra and continued to match the sample spectra and

database spectra by using Biotyper Real Time Classification software v3.1 (Bruker, USA) for microbial identification (Pérez-Sancho, et al. 2018). The characterization of *A. schubertii* by was undergone by using MALDI-TOF MS spectra measured by Bruker Daltonics UltrafleXtreme MALDI-TOF MS machine and MALDI-TOF MS Biotyper® library. After done spectra preprocessing parameters including mass adjustment, smoothing, normalization, peak detection, baseline correction and outlier removal by using Bruker Daltonics FlexAnalysis® software, the spectra of each isolate were undergone for spectra stacking, principal component analysis and dendrogram of relatedness by using Bruker Daltonics Clinprotool® software. And then, main spectra profile was built from each isolate by using Bruker Daltonics MBT Biotyper® software and in-house library was constructed future *A. schubertii* identification (Murugaiyan et al., 2018). *Aeromonas sp.* used in this part as out group are explained in the Appendix of this thesis.

3.3 Pathogenicity assay

3.3.1 Bacterial preparation

A single colony of *A. schubertii* was grown on TSA will be cultured in LB broth (BD Difco™, New Jersey, USA) and incubated in a shaking incubator at 150 rpm for 24 h at 28°C. Next, the bacterial solution was centrifuged at 10.000 ×g for 15 min. And then, the bacterial pellet was resuspended in phosphate buffered saline 1x (PBS 1x) to obtain 10^9 CFU ml^{-1} concentration in spectrophotometry OD_{600} of ~0.8. The

actual number of bacteria was determined by drop plate method using 50 µl of each 10-fold diluted sample on TSA and counting the resulting colonies after 24 h incubation at 28°C.

3.3.2 Experimental fish

The two hundred ten healthy striped snakehead fish fingerlings were obtained from snakehead fish hatchery, Nakhon Pathom province, Thailand. The estimated weight and length of the fish was ±5g and ±10cm, respectively. The fish was acclimated for 7 days in 1000 L water tank with aeration before experimental challenges. ten representative fish of the population was confirmed as negative for *Aeromonas* sp. by culturing liver samples in blood agar plates, incubated at 28°C for 24 h. Samples was also examined parasite infection by wet mount method.

3.3.3 Experimental challenge to determine pathogenicity of *A. schubertii* in striped snakehead fish

To investigate pathogenicity of this pathogen to striped snakehead fish, an experimental design was created to challenge the *A. schubertii* isolate of this study (CUVETASC03) into striped snakehead fish fingerlings through intraperitoneal injection with infected fish.

The experimental design was used two hundred ten fish designed as seven groups. Six groups of fish (15 fish per group and duplicated) were injected via the intraperitoneal route (IP) with 0.1 ml different bacteria doses 10^3, 10^4, 10^5, 10^6, 10^7,

and 10^8 CFU $fish^{-1}$ and the non-challenge group or control group was injected with 0.1 ml sterile PBS intraperitoneally. After injection, each group of fish were transferred to related group. The fish were anesthetized by immersing in 60 mg/L clove oil before being injected with dose of *A. schubertii* (Park, 2019).

The experiment was observed two times a day for 21 days and recorded. The water conditions (pH, diluted oxygen, ammonia and temperature) were evaluated and the 38% protein commercial pellet (CP, Thailand) was given 2 times a day, simultaneously.

3.3.4 Data Recording.

The data recordings were collected two times in each day and accumulated as 7-, 14-, and 21-days post injection (dpi). The freshly dead fish (maximum 2 hr post-mortem) in each day were collected and necropsied aseptically. Then, it was inspected for gross pathology lesions (skin ulceration, internal organ hemorrhage and white nodules). The gross pathology lesions were recorded as semi-quantitative data as followed: 0 – no visible change, 1 – mild changes, 2 – moderate changes, and 3 – severe changes. The detail of semi-quantitative recording of gross pathology lesion was explained in this thesis appendix. Moreover, these freshly dead fish as well as were counted as mortality to obtain cumulative mortality in 7, 14, and 21 dpi.

The inspected internal organs of each specimen were cultured on TSA supplemented with sheep blood 5% to recover the bacteria colony of CUVETASC03. Then, it was re-identified by using primary biochemical assays (gram staining, oxidase, and catalase) and MALDI-TOF MS.

The internal organs (liver, kidney, and spleen) of necropsied dead fish were and preserved in 10% buffered formalin. Subsequently, the samples were undergone for histopathology slides and stained by using hematoxylin and eosin (H&E) staining. The process was done as followed: Each of internal organ was dehydrated by using different concentration of ethanol (75, 80, 85, 90, and 95%) and xylol, embedded in paraffin block, sectioned at thickness 5 µm into slides, and stained with hematoxylin and eosin. The histopathology slides of internal organ were observed under a microscope attached with a digital camera and recorded as JPG file. The histopathology lesions (granuloma, melano-macrophage center (MMC) and necrotic cells) were observed in each of histopathology slides of each internal organs (liver, kidney, and spleen) and recorded as semi-quantitative data as followed: 0 – no visible change, 1 – mild changes, 2 – moderate changes, and 3 – severe changes. The detail of semi-quantitative recording of histopathology lesions was explained in this thesis appendix.

3.4 Data Analysis

The dead fish which had been recorded were undergone to median lethal dose (LD_{50}) determination. The LD_{50} was deterimined by using Reed and Muench (1938) method. The formula of Reed and Muench (1938) shows as followed :

$$\frac{\text{50 percent – (mortality of next below)}}{\text{(Mortality of next above) – (mortality of next below)}} = \text{Proportional distance}$$

Log (lower dilution) + ((proportional distance) x Log (dilution factor)) = sum (log of end point)

Figure 2. Formula of Reed and Muench

The significant differences were calculated among cumulative mortality, gross pathology lesions (skin ulceration, internal organ hemorrhage, white nodules) and histopathological lesions (granuloma, MMC and necrotic cells) in each treatment groups (10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 CFU fish-1) and in each of 7, 14, and 21 dpi by using Kruskal-Wallis test of SPSS® 22 software package (IBM, USA) and continued by using Man Whitney U test as mean comparison test with P value < 0.05.

CHAPTER IV RESULTS

4.1 Isolation, Identification and Characterization.

4.1.1 Isolation and Identification.

The pre-dominant colonies were isolated from internal organs of diseased striped snakehead fish (*Channa striata*) fingerlings (approximately 3 weeks-old) with ±5 g of bodyweight and ±5 cm of length from Nakhon Pathom province in July 2020. The fish showed darkening skin, skin hemorrhage and ulcers and lethargy. Then, these isolates were initially identified by utilization of biochemical assay and MALDI-TOF MS. It was obtained the most pre-dominant colonies were identified as *A. schubertii* (6 isolates), which is afterward labeled as CUVETASC03, CUVETASC04, CUVETASC05, CUVETASC06, CUVETASC07, and CUVETASC08. In addition, histopathology observation of internal organs of natural infected snakehead fish had been conducted to observed histopathology lesions from natural infection. The initial identification by using biochemical assay and MALDI-TOF MS, gross pathology and histopathology observation of natural infected striped snakehead fish can be seen in Appendix of this thesis.

4.1.2 Biochemical profile Characterization by using Biochemical Assay.

The biochemical profile characterizations of six isolates of *A. schubertii* evaluated by using biochemical assays and Vitek-2 compact® were successfully conducted. Even though, all *A. schubertii* isolates biochemical profile had been

successfully evaluated by Vitek-2 compact® GN card. Vitek-2 compact® was unable to identify the species of these isolates. the details of Vitek-2 compact® GN card results were describes in this thesis appendix. In this biochemical evaluation, all these isolates had identical results compared to published data of biochemical profile of reference *A. schubertii*. The comparative biochemical profile between this study isolates and reference isolates were shown in table 5. Moreover, these isolates seemingly are different characteristics compared to reference *A. hydrophila*. Therefore, *A. schubertii* can be distinguished on some biochemical characteristic among other *Aeromonas* genus, such as indole, VP, esculin hydrolysis, gluconate test, arabinose, lactose, and sucrose fermentation. In addition, there were inconsistent results among this study *A. schubertii* isolates, i.e. methyl-red, ornithine decarboxylase and gelatin hydrolysis result. Likewise, it was compared to the reference *A. schubertii* isolates and they had similar inconsistent results among the reference isolates as well.

Table 4. Phenotypic characteristic of this study *A. schubertii* isolates compared with reference isolates.

Characteristics	*A. schubertii* this study (n=6)	Reference isolates				
		A. schubertii (Liu & Li, 2019) (n=9)	*A. schubertii* (Chen et al, 2012) (n=4)	*A. schubertii* (Hickman-Brenner et al., 1988) (n=6)	*A. schubertii* (Abbot & Janda, 2006) (n=12)	*A. hydrophila* (Abbot & Janda, 2006) (n=25)
Gram stain	-	-	ND	ND	ND	ND
Shape	SR	ND	ND	ND	ND	ND
Oxidase	100	100	100	100	ND	ND
Catalase	100	100	100	ND	100	100
Hemolysin	100	100	ND	71	75	75
Motility	100	100	100	100	100	100
Oxidative/Fermentative	F	F	F	F	ND	ND
Indole	0	0	0	0	8	90
Methyl Red (MR)	83.33	100	100	100	ND	ND
Voges Proskauer (VP)	0	0	25	0	12	93
Arginine deaminase	100	100	0	86	92	100
Lysine decarboxylase	100	100	25	100	83	100
Ornithine decarboxylase	16.67	0	25	0	0	0
Esculin hydrolysis	0	0	0	0	0	92
Nitrate reduction	100	100	100	100	ND	ND

Characteristics	*A. schubertii* this study (n=6)	Reference isolates				
		A. schubertii (Liu & Li, 2019) (n=9)	*A. schubertii* (Chen et al, 2012)	*A. schubertii* (Hickman-Brenner et al., 1988) (n=6)	*A. schubertii* (Abbot & Janda, 2006) (n=12)	*A. hydrophila* (Abbot & Janda, 2006) (n=25)
Gluconate	0	0	0	ND	0	64
Citrate Simmons	0	ND	0	29	58	88
Urease	0	ND	66.67	0	0	0
Phenylalanine	0	100	0	57	67	60
H2S production	0	0	0	0	ND	ND
Malonate utilization	0	ND	ND	0	0	0
Maltose	100	100	100	100	100	100
Arabinose	0	0	0	0	0	84
Sucrose	0	0	0	0	0	100
Lactose	0	0	0	0	0	16
Adonitol	0	ND	0	0	0	0
Trehalose	0	100	ND	100	ND	ND
Mannitol	0	0	0	0	0	96
Mannose	0	ND	100	100	92	100
Sorbitol	0	0	0	0	0	0
Cellobiose	0	0	0	0	0	0
α-CH_3-D- glucoside	0	ND	ND	0	0	56
Xylose	0	0	0	0	ND	ND
Galactose	100	ND	100	0	ND	ND
Tyrosine	100	ND	ND	29	8	0
ONPG test	66.67	0	ND	86	83	100

Characteristics	*A. schubertii* this study (n=6)	Reference isolates				
		A. schubertii (Liu & Li, 2019) (n=9)	*A. schubertii* (Chen et al, 2012)	*A. schubertii* (Hickman-Brenner et al., 1988) (n=6)	*A. schubertii* (Abbot & Janda, 2006) (n=12)	*A. hydrophila* (Abbot & Janda, 2006) (n=25)
O/129 resistance	100	ND	ND	0	100	100
Casein	100	ND	ND	100	83	83
Gelatin hydrolisis	0	100	ND	0	ND	ND
Lecithinase	100	ND	ND	100	92	100
Olive oil	0	ND	ND	0	ND	ND
Glycerol	0	0	ND	0	0	96
NaCl Conc. 0.5%	100	100	ND	100	92	100
NaCl Conc. 1%	100	100	ND	100	100	100
NaCl Conc. 1.5%	100	ND	ND	ND	100	100
NaCl Conc. 2%	100	ND	ND	ND	100	100
NaCl Conc. 2.5%	100	ND	ND	ND	100	100
NaCl Conc. 3%	100	ND	ND	ND	ND	ND
NaCl Conc. 3.5%	100	ND	ND	ND	ND	ND
NaCl Conc. 4%	0	ND	ND	ND	ND	ND
NaCl Conc. 4.5%	0	ND	ND	ND	ND	ND
NaCl Conc. 5%	0	ND	ND	ND	ND	ND

*The results were interpreted after 24h incubation at 28°C and it is considered as percentage of positive result, **-:** gram negative, SR: short rod, F: Fermentative, Conc.: concentration, ND: no data available.

4.1.3 PCR assay, DNA sequencing, and Phylogenetic Tree by based on 16S rRNA and *gyrB* sequences.

All of DNA of the *A. schubertii* isolates had been successfully extracted by using Queipo-Ortuño et al. (2008)'s boiling method. Then, it was amplified by using PCR assay based on 16S rRNA and *gyrB* sequences. The amplicons were approximately 1500 bp for 16S and 1000 bp for *gyrB* sequences. It can be seen on figure 3. Moreover, all of the isolates were sequenced by using Sanger sequencing and blasted through NCBI nucleotide database by using BLASTn in both sequences (16S rRNA and *gyrB*). Regardless, it showed that all of the isolates shared 99.6%-99.9% similar identity with published *A. schubertii* strains. The details can be shown on table 6. The maximum likelihood phylogenetic tree had been constructed based on ±1500 bp of 16s rRNA sequences and ±1000 bp of *gyrB* sequences compared with other *A. schubertii* strains obtained from GenBank and other bacteria as out-group. All of the *A. schubertii* of this study (CUVETASC03, CUVETASC04, CUVETASC05, CUVETASC06, CUVETASC07, and CUVETASC08) were clustered with reference *A. schubertii* strains. It showed on both 16S rRNA and *gyrB* sequence phylogenetic trees. The phylogenetic trees can be seen in figure 4.

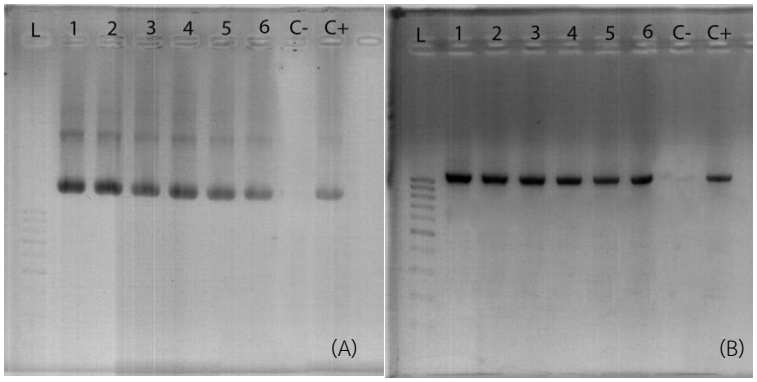

Figure 3. PCR Amplification of 16S rRNA (A) and *GyrB* (B) sequences of *A. schubertii* in this study visualized on 1.5% agarose gel. Lane L = Marker ladder, lane 1 = CUVETASC 03, lane 2 = CUVETASC04, lane 3 = CUVETASC05, lane 4 = CUVETASC06, lane 5 = CUVETASC07, lane 6 = CUVETASC08, lane C- = negative control, lane C+ = positive control.

Table 5. Details of Blasted *A. schubertii* isolates by using 16S rRNA and *gyrB* sequences

Isolate	16S rRNA sequences				*gyrB* sequences			
	Most closely related species	query coverage	identity (%)	accession No.	Most closely related species	query coverage	identity (%)	accession No.
CUVETASC 03	*A. schubertii* WL-1	100	99.71	JQ303346.1	*A. schubertii* HYL1	100	99.80	HQ731454.1
CUVETASC 04	*A. schubertii* LF1708	100	99.70	MG593845.1	*A. schubertii* HYL1	100	99.75	HQ731454.1
CUVETASC 05	*A. schubertii* LF1708	100	99.70	MG593845.1	*A. schubertii* WL-4	100	99.75	MK416159.1

Isolate	16S rRNA sequences	gyrB sequences

	Most closely related species	query coverage	identity (%)	accession No.	Most closely related species	query coverage	identity (%)	accession No.
CUVETASC06	*A. schubertii* LF1708	100	99.68	MG593845.1	*A. schubertii* HYL1	100	99.80	HQ731454.1
CUVETASC07	*A. schubertii* LF1708	100	99.70	MG593845.1	*A. schubertii* WL-4	100	99.80	MK416159.1
CUVETASC08	*A. schubertii* LF1708	100	99.70	MG593845.1	*A. schubertii* WL-4	100	99.75	MK416159.1

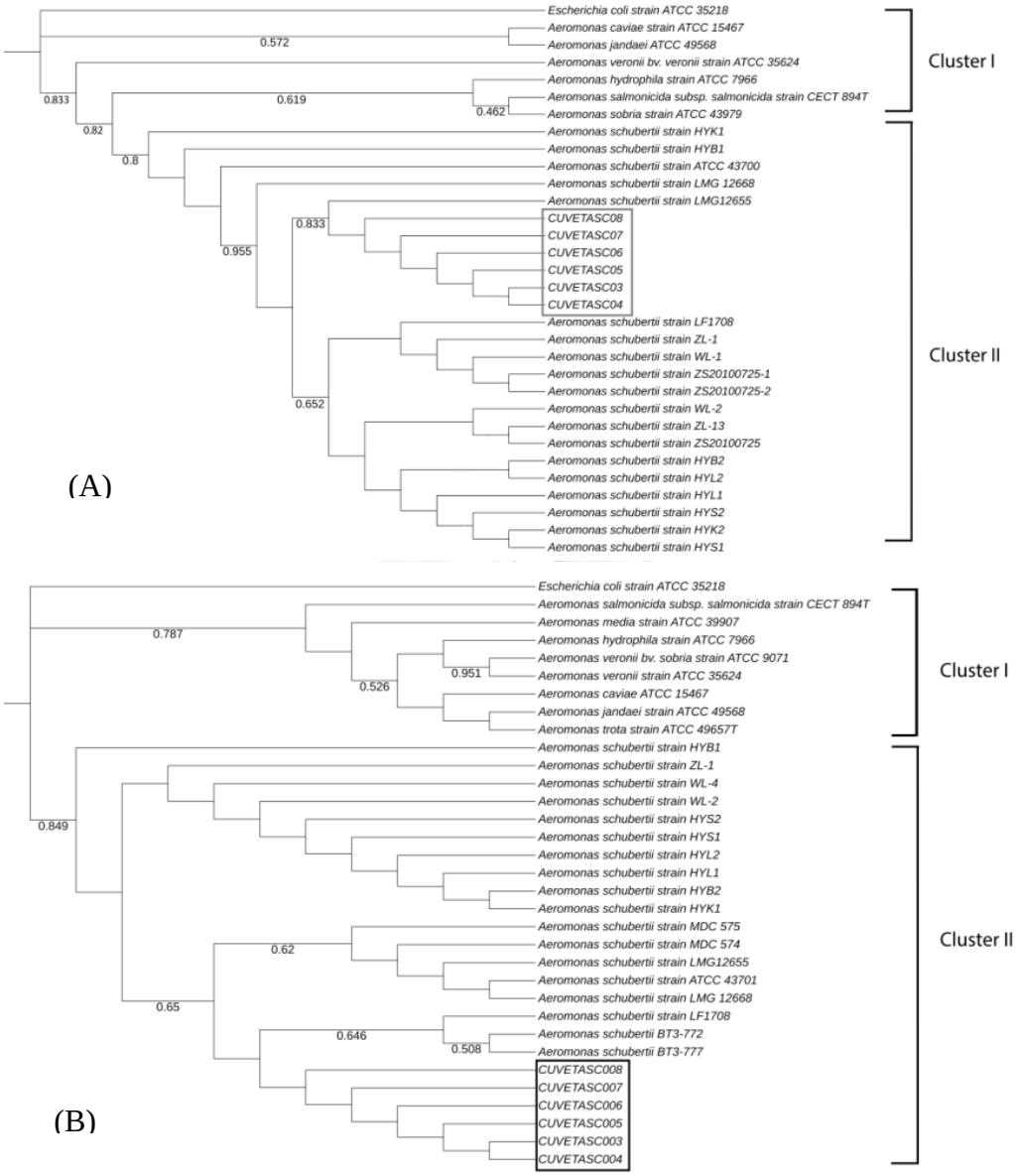

Figure 4. Maximum likelihood phylogenetic tree constructed based on 16S rRNA sequences (A) and based on *gyrB* sequences (B). The cluster I was out-group *Aeromonas spp.* and *E. coli* ATCC 35218 and the cluster II was group of *A. schubertii*. Black rectangle is group of *A. schubertii* which are used in this study. Numbers showed the bootstraps value.

4.1.4 Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry.

The six isolates of *A. schubertii* (CUVETASC03, CUVETASC04, CUVETASC05, CUVETASC06, CUVETASC07 and CUVETASC08) and four other isolates (*A. hydrophila*, *A. veronii*, *A. jandaei* and *A. caviae*) as an out-group had been extracted and successfully measured in the MALDI-TOF MS equipment (Bruker Daltonics, Massachusetts, USA). Spectrum preprocessing parameters on every isolate, such as peak detection, baseline correction, spectrum smoothing and normalization, and outlier removal had been conducted. Spectra view, principal component analysis (PCA) and dendrogram based on spectra produced by MALDI-TOF had been constructed and it can be seen in figure 5, 6 and 7.

The spectra view of each isolates including out-group isolates seemingly had different pattern of biopolymer detected by MALDI-TOF MS. Moreover, the *A. schubertii* isolates spread and can be clustered into one area in PCA view. The clustering can be seen on the other isolates as well. In dendrogram view, The *A. schubertii* of this study were clustered into one cluster group compared with other isolates.

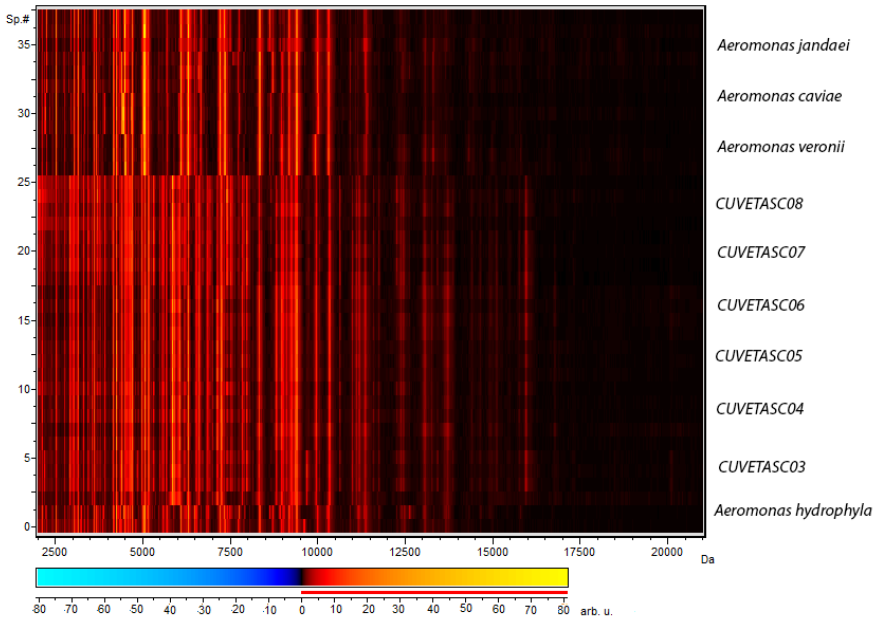

Figure 5. spectra view on gel stack view of *A. schubertii* and other *Aeromonas spp.* isolates.

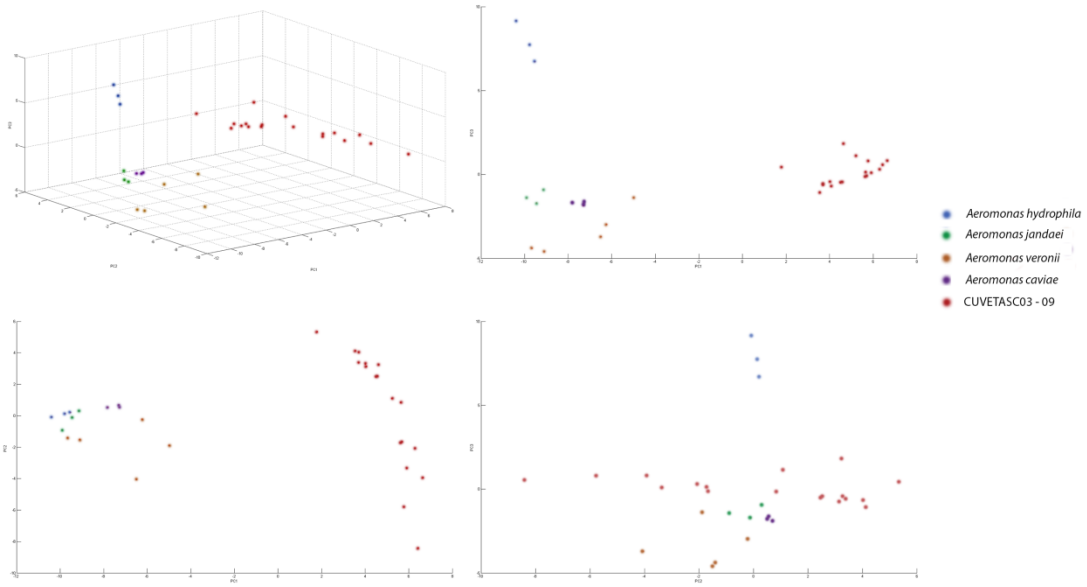

Figure 6. Principle of component analysis (PCA) of *A. schubertii* of this study and other *Aeromonas* sp. isolates.The isolates are clustered in red colored dots are *A. schubertii* used in this study. Other colored dots are out-group *Aeromonas* sp. isolates.

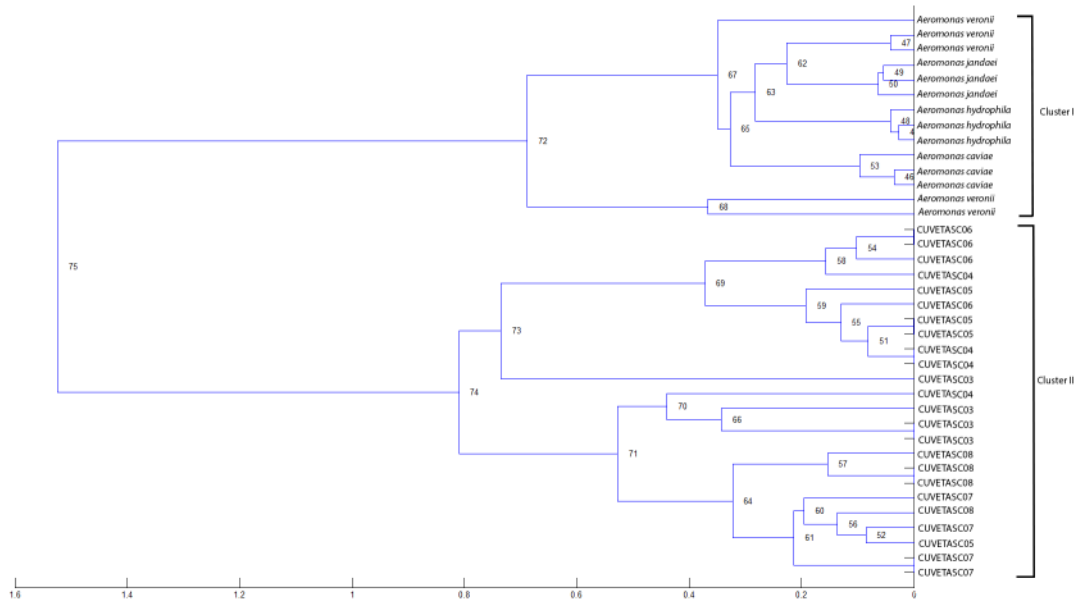

Figure 7. Dendrogram of likelihood of *A. schubertii* of this study and other *Aeromonas* sp. isolates. The Cluster I are included out-group of *Aeromonas* sp. isolates and cluster II are included *A. schubertii* isolates of this study.

4.2 Pathogenicity

4.2.1 Cumulative mortality rate and median lethal dose (LD_{50}) determination.

The experimental challenge was conducted by using CUVETASC03 isolate as the pathogen injected to the 7 groups of striped snakehead fish ($1.2x10^3$, 10^4, 10^5, 10^6, 10^7, 10^8 and control). After recording 21 days, it obviously produced mortality to striped snakehead fish. The clinical sign on lowest dose ($1.2x10^3$) showed skin ulcers, internal organ hemorrhage and white spot nodules on 7 days post injection (PI). In addition, solitary behavior and reduced feed intake were observed on the first day PI.

From collected experimental fish specimens, the *A. schubertii* were able to be isolated and recovered.

The cumulative mortality rate, distribution, and median lethal dose (LD_{50}) of CUVETASC03 isolate in this study had been calculated. The LD_{50} was determined by using Reed and Muench (1938)'s formula. It showed LD_{50} was on 3.9×10^5 dose of *A. schubertii*. The calculation of LD50 can be seen on the Appendix of this thesis.

On the other hand, the cumulative mortality distribution was calculated based on the mortality rate of striped snakehead fish injected by different dose of *A. schubertii* (CUVETASCO3) on 7-, 14-, and 21-days post injection (dpi) and on each dosage. By using one-way analysis of variance (ANOVA) and Duncan post hoc test with p-value < 0.05, the cumulative mortality showed significances on higher dose ($1.2x10^5$, 10^6, 10^7 and 10^8) compared to lower dose ($1.2x10^3$ and $1.2x10^4$) and control group on 7-dpi, 14-dpi or even 21 dpi. Even though, lowest dose showed insignificances from first 7 days until 14 days PI, it formed significance after 14-dpi until 21-dpi. The details of cumulative mortality distribution can be seen in figure 8.

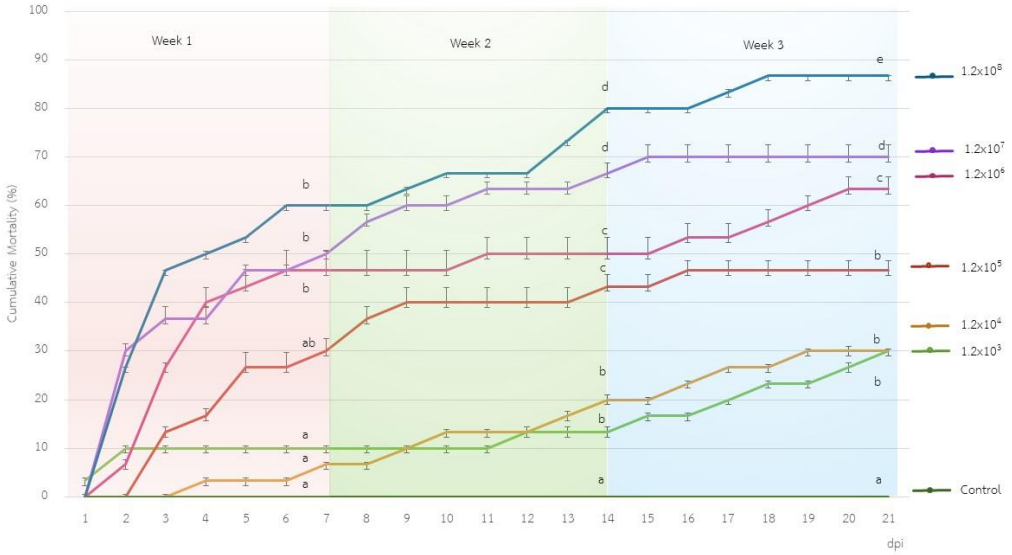

Figure 8. Diagram of cumulative mortality distribution (mean ± SD) of striped snakehead fish injected by different dose of CUVETASC03 study within 7-, 14-, and 21- dpi. The superscript letters (^{a}, ^{b}, ^{c}, ^{d}, and ^{e}) are indicative of different significance and similar superscript letter is indicative of insignificance analyzed by using one-way ANOVA and continued by using Duncan post hoc test as mean comparison with p-value < 0.05. “week 1”: 7-dpi, “week 2”: 14-dpi, “week 3”: 21-dpi.

4.2.2 Gross pathology and histopathology Analysis

The experimental fish specimens had been collected dead in each group on 7-, 14-, and 21-days post injection (dpi). Then, the clinical signs, both gross pathology and histopathology lesions, were observed. The gross pathology lesions (skin ulceration, internal hemorrhage, and white nodules) had been observed, scored, and recorded in each fish specimen. It can be seen in figure 9.

To observe the significances between gross pathology lesions in each day ost injection and different dose, scored gross pathology lesions (white nodules, skin

ulcerations, and internal hemorrhage) had been calculated and analyzed by using Kruskal-Wallis test and continued by using Mann-Whitney U test as mean comparison with p-value < 0.05. it showed that the skin ulcerations did not show any significances between 7-, 14-, and 21dpi, but significances were able to be seen In internal hemorrhage at 21-dpi as similar as white nodules in liver. Moreover, there were significance in white nodules either in kidney or spleen. both had significance of white nodule score between 7-,14- and 21-dpi. It can be seen in figure 11 (A).

On the other hand, the significances of gross pathology lesions between groups of different doses of *A. schubertii* showed scored white nodules in liver, kidney and spleen, skin ulcerations, internal hemorrhages were significantly different between control group and other groups. the Group of doses $1.2x10^8$ of skin ulceration, internal hemorrhage, white nodules of kidney and spleen were significant different to other groups of doses ($1.2x10^3$, 10^4, 10^5, 10^6 and 10^7). However, the groups of doses $1.2x10^3$, 10^4, 10^5, 10^6, and 10^7 in scored kidney white nodules, spleen white nodules, skin ulceration and internal hemorrhage had insignificance among the groups. It was similar occurrence in liver white nodules which it was significant between control and other groups of doses, but it was insignificance among groups of doses including group $1.2x10^8$. The Data of observed gross pathology lesions among group of *A. schubertii* dose can be seen on figure 12 (A).

The histopathology observation of each internal organ (liver, kidney, and spleen) of each specimen had been conducted under microscopes. It seemed that the histopathology lesions between those organs and timeline based shared similar pattern. On the 7-dpi, cell nucleus alterations had occurred in hepatocytes, tubule cells and pancreatic cells, but it did not alter the cytoplasmic vacuoles, cytoplasm, cell membrane or even the overall tissue itself. It can be seen as similar as the control. Moreover, the melano-macrophage centers (MMCs) began occurring on kidney and spleen on 14-dpi. The tissue alteration such as fibrosis, hemorrhage, aneurysm, and loss integrity intra- and inter-cellular on the interstitial tissue apparently occurred in this period. Poly mononuclear cell infiltration were also visible in all observed organs at this period time. In 21-dpi, visible granulomas were completely formed in all organs, and infiltration of various white blood cells were obviously occurred in all observed internal organs. Accumulation fibrin tissue in spleen and necrotic cells (necrotic hepatocytes in liver, necrotic tubule, and necrotic bowman cells in kidney) were visible in all possible view of histopathology. The MMCs began to develop in liver but the other organ's MMCs were manifested intensely in this period. the histopathology observations can be seen in figure 10.

Then, The Histopathology lesions (granulomas, melano-macrophage centers (MMCs) and necrotic cells (NCs)) were scored and calculated as well as analyzed by using Kruskal-Wallis test and Mann-Whitney U test as mean comparison with p-value

< 0.05. the analysis showed significances on liver, kidney, and spleen lesions. Granuloma, MMC and NC in liver showed significances on 7-, 14-, and 21-dpi. It was similar occurrence on kidney granuloma, MMCs and NCs. However, MMCs of spleen seemingly showed in significance on 14- and 21-dpi, even though it showed significance on 7-dpi. Granuloma and NC on the spleen was similar with other organ, the significance occurred on 7-, 14-, and 21-dpi. The histopathology lesions analysis of each organ (liver, kidney, and spleen) can be seen in figure 11 (B), (C), and (D), respectively.

The analysis of histopathology observation between different dose of *A. schubertii* had been conducted and summarized. It showed that control group of each internal organ (liver, kidney, and spleen) histopathology lesions (granuloma, MMCs, and NCs) were significantly different with other group ($1.2x10^3$, 10^4, 10^5, 10^6, 10^7, and 10^8). Likewise, the $1.2x10^8$ group of liver granulomas, liver NCs, kidney granulomas, kidney MMCs, spleen granulomas, spleen MMCs, and spleen NCs were also showed significance to other groups of doses of *A. schubertii*. while liver MMCs and kidney NCs were not significantly different among groups of doses. The group of $1.2x10^3$, 10^4, 10^5, 10^6, and 10^7 were not showed any significance in all internal organ histopathology lesions which were observed. the detail can be seen in figure 12 (B), (C), and (D).

Moreover, data analysis of comparison between score of observed white nodules of gross pathology and granuloma of histopathology in each internal organ (liver, kidney, and spleen) had been computed by using Mann-Whitney U test. it revealed that there were different significances were occurred on 7-, 14-, or 21-dpi in both liver white nodules and granuloma, yet kidney white nodules and granulomas only showed significance on the 14-dpi and 21-dpi. However, non-significances were shown between white nodules and granuloma of spleen at all time periods. The detail can be seen in figure 13.

The correlation between both white nodules of gross pathology and granuloma of histopathology scores of observations in each internal organs had been successfully calculated by using Spearman correlation bivariate test with p-value 0.05 to see whether both has strong relationship or not. The result of correlation analysis showed that the liver, kidney and spleen white nodules and its granuloma had 0.510, 0.826 and 0.770 correlation coefficient, respectively. the correlation analysis was interpreted as liver white nodules has weak positive correlation to liver granulomas. However, kidney white nodules of gross pathology had strong positive correlation to kidney granulomas. Likewise, spleen had similar interpretation as kidney. It can be interpreted as spleen white nodules has strong positive correlation to spleen granuloma. Correlation analysis by using Spearman test with p-value < 0.05 can be seen in this thesis appendix.

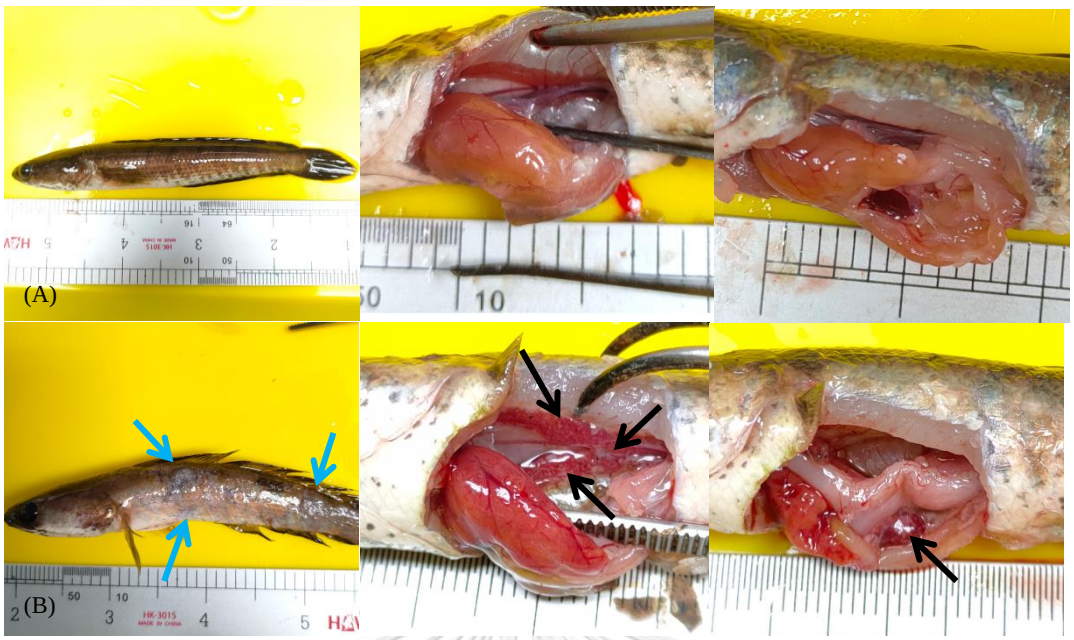

Figure 9. Observed gross pathology lesions caused by *A. schubertii* on experimental challenge. The upper pictures (A) are control group and lower pictures (B) are treatment group. Blue arrows indicate skin ulcers and black arrows indicate white nodules.

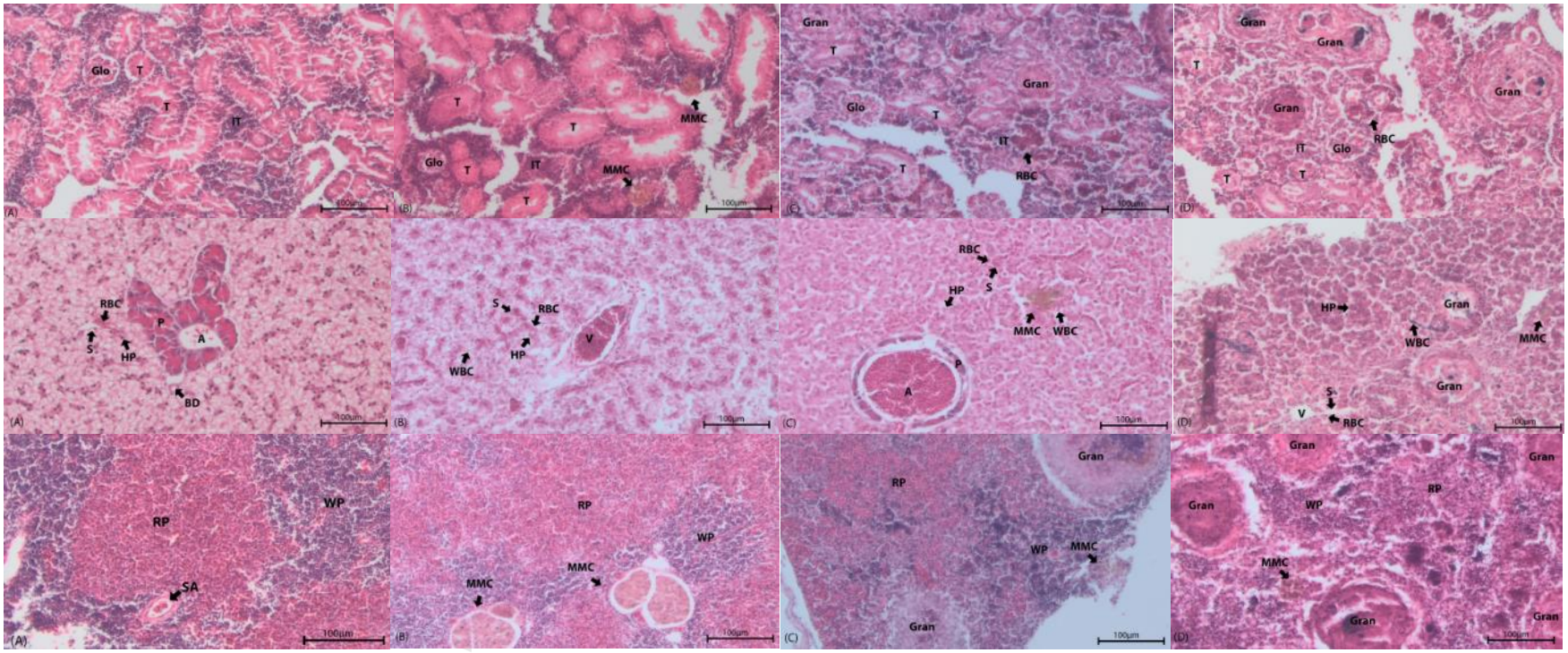

Figure 10. Histopathology lesions caused by *A. schubertii* on experimental challenge were observed under microscope 100x magnifications. The upper pictures are kidney histology view, middle pictures are liver histology view and lower pictures are spleen histology view. The (A) pictures are control group, (B) pictures are 7-days post injection (PI), (C) pictures are 14-days PI and (D) pictures are 21 days PI. Glo: glomerulus, T: tubules, IT: interstitial tissue, HP: hepatocytes, S: sinusoid, P: pancreas, BD: bile duct, A: artery. V : Vena porta, RP: red pulp, WP: white pulp, SA: splenic arteriole, RBC: Red blood cells, WBC: white blood cells, MMC: melano-macrophage center and Gran: Granuloma.

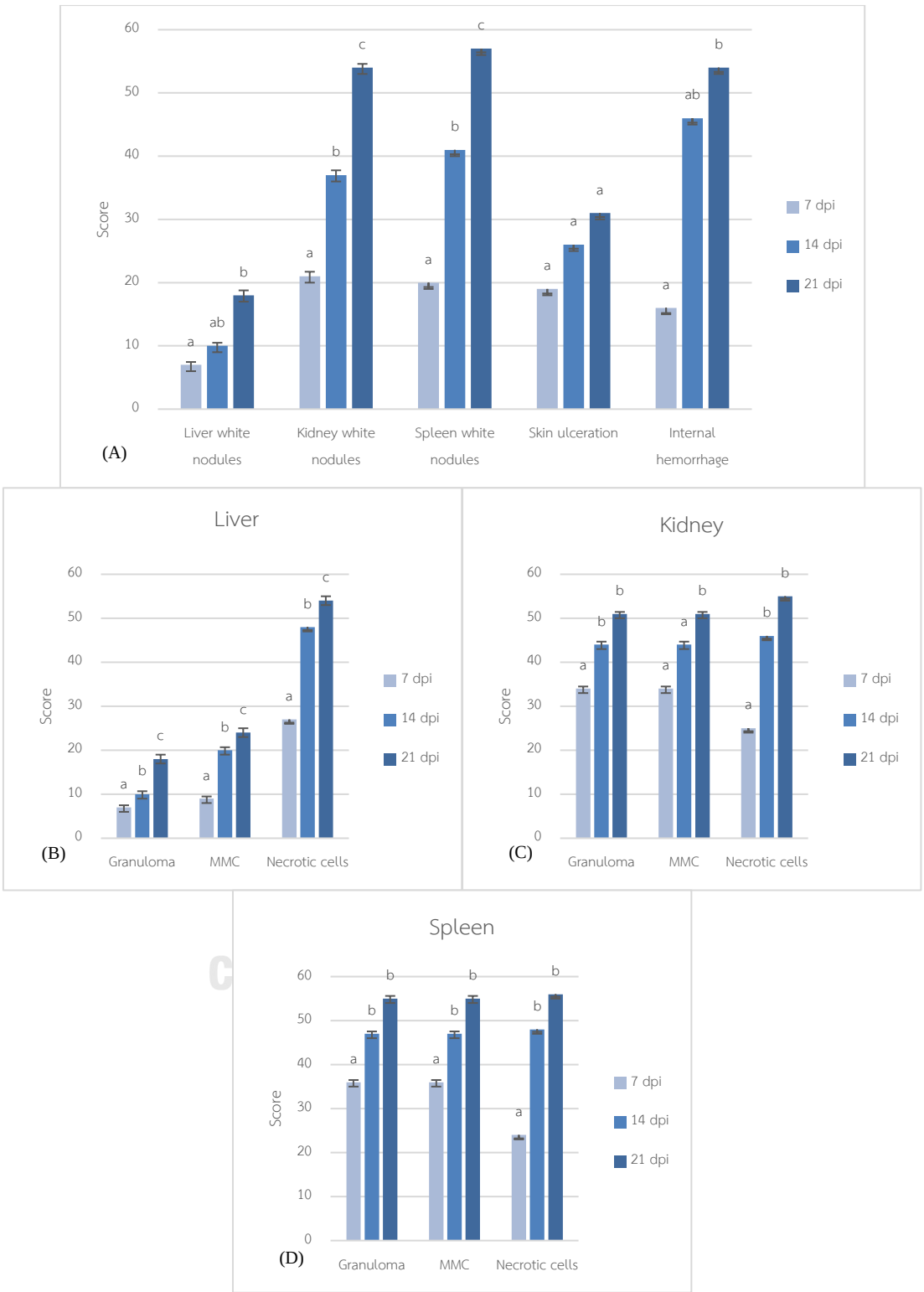

Figure 11. Comparative diagrams of gross pathology lesions histopathology and lesions semi-quantitative score of each internal organs (liver, kidney, and spleen) of fish injected with CUVETASC03 within 7-,14-, and 21-days post injection (dpi). Diagram (A) was graph of gross pathology lesions (white nodules in internal organs (liver,

kidney, and spleen), skin ulceration, and internal hemorrhage. the (B), (C), and (D) were graph of score of histopathology lesions (granuloma, MMC, and necrotic cells) observed in liver, kidney, and spleen under light microscope. The superscript letters (^{a}, ^{b}, and ^{c}) were indicative of different significance group and similar superscript letters were indicated of insignificance. It had been analyzed by using Kruskal-Wallis test and continued by using Mann-Whitney U test as mean comparisons with p-value < 0.05.

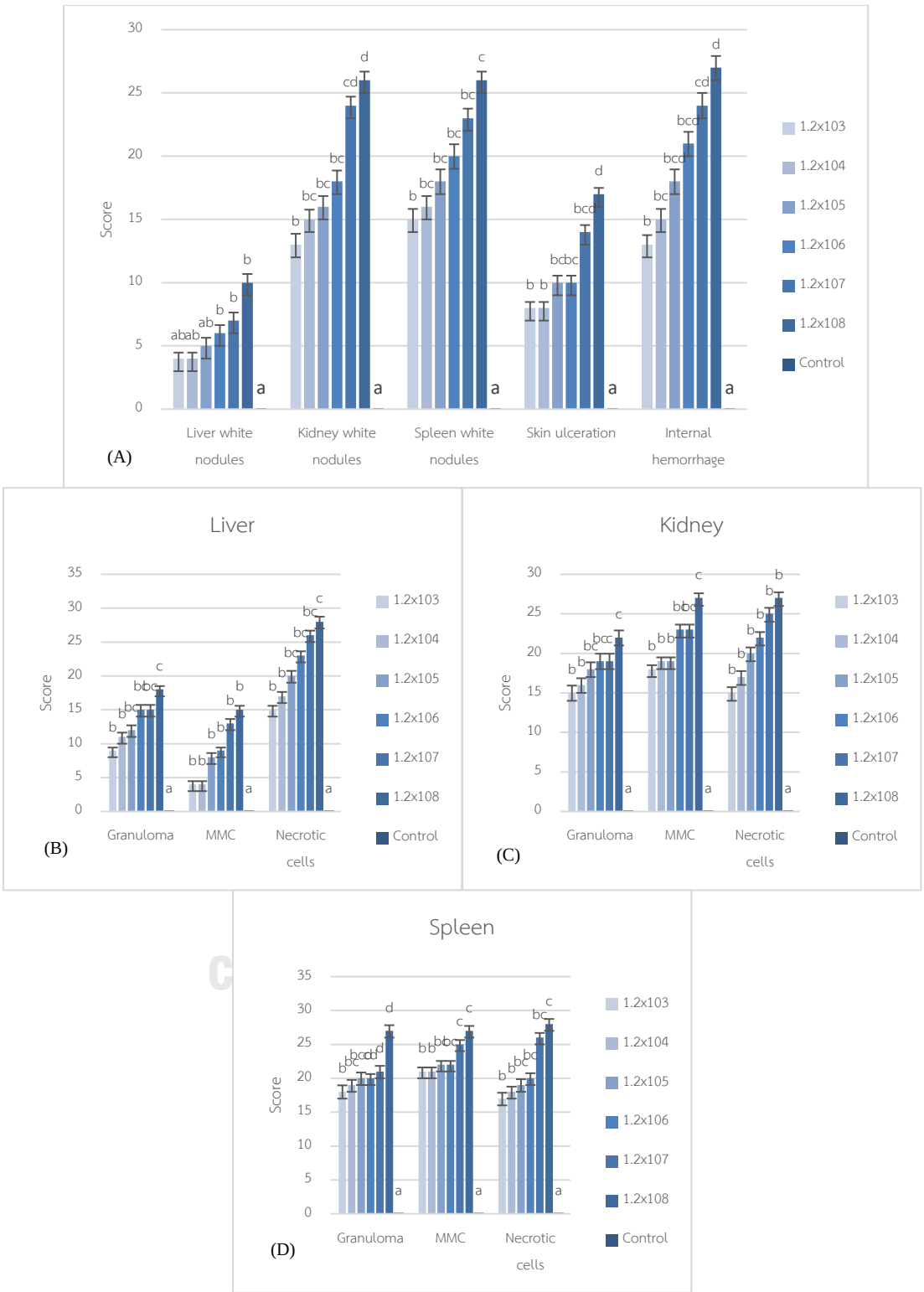

Figure 12. Comparative diagrams of gross pathology lesions histopathology and lesions semi-quantitative score of each internal organs (liver, kidney, and spleen) of fish injected with different dosage of *A. schubertii* (CUVETASC03). Diagram (A) was graph of gross pathology lesions (white nodules in internal organs (liver, kidney, and

spleen), skin ulceration, and internal hemorrhage. the (B), (C), and (D) were graph of score of histopathology lesions (granuloma, MMC, and necrotic cells) observed in liver, kidney, and spleen under light microscope. The superscript letters ([a], [b], [c], and [d]) were indicative of different significance and similar superscript letters were indicative of insignificance. It had been analyzed by using Kruskal-Wallis test and continued by using Mann-Whitney U test as mean comparisons with p-value < 0.05.

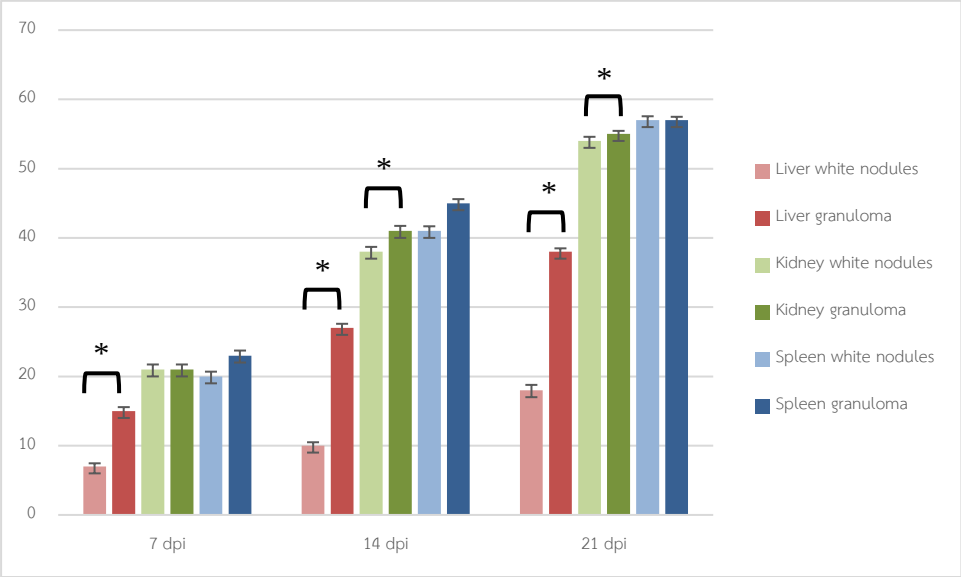

Figure 13. Comparative diagram of white nodules of gross pathology and granuloma of histopathology in each internal organs (liver, kidney, and spleen) within 7-,14-, and 21-dpi. The asterisk (*) were indicated as different significance of both compared groups analyzed by using Mann-Whitney U test with p-value < 0.05.

CHAPTER V DISCUSSIONS AND CONCLUSIONS

5.1 Discussion.

An unknown outbreak in striped snakehead fish (*Channa striata*) farm in Nakhon Pathom province, Thailand in July 2020 was occurred and investigated comprehensively to identify the causative agent. By utilizing initial combined identification process, such as biochemical assay, PCR and DNA sequence (16S rRNA and g*yrB*) as well as MALDI-TOF MS assay, the presumptive pre-dominant colonies isolated from internal organs of the fish was *A. schubertii*. The clinical signs from striped snakehead fish fingerlings which was collected from the outbreak site were high mortality, lethargy, skin ulceration and internal hemorrhage. This case was similar to an *A. schubertii* infection happened in Hubei province, central China and Zhongshan, southern China in 2009. Both showed high mortality, skin ulceration and internal hemorrhage in blotched snakehead fish (Channa maculate) (Chen et al., 2012) and northern snakehead fish (Channa argus) (Liu and Li, 2012), respectively. Special feature in this infection was white nodules which is related to *Mycobacterium* sp., *Nocardia* sp*.,* or *Francisella* sp. infections (Liu and Li, 2012). This outbreak also showed manifestation of white nodules in internal organs such as liver, kidney, and spleen. in this case, it needed further investigation on histopathology observation.

Our finding on characterization of *A. schubertii* isolates by using conventional biochemical assays and Vitek-2 compact® GN CARD showed identical results among *A. schubertii* reference. comparing with *A. hydrophila* as reference, they had similar in some part of characteristics because they share same family and genus. it may share similar characteristics, such as fermentative behavior, oxidase test positive, does not requires NaCl to grow, fermenting maltose and nitrate reducing to nitrite, which is belongs to *Aeromonas* family (Hickman-Brenner et al., 1988). However, NaCl tolerant is commonly found in *Aeromonas* family. A report said *Aeromonas* spp. may be able to grow up to 1M or 5.8% NaCl concentration yet it is slower in grow (Delamare et al., 2000). Modification of sodium transportation and reducing osmotic cell membrane stressor may allow *Aeromonas* spp. adapt on high concentration of NaCl (Wood et al., 2001). Thus, it may cause confusion to identify between *Aeromonas* spp. and *Vibrio* spp. which is also high NaCl tolerant (Delamare et al., 2000). The Vitek-2 compact® GN CARD had been reported by Lamy et al. (2011). They said the inadequate database and inaccurate logarithm were clearly undermined the performance of the Vitek-2 systems (Lamy et al., 2011). Even though *A. schubertii* was added to Omnilog systems and MicroScan walkaway, it was beneath good performance of the system to identify this species because of the confusion of *Aeromonas* taxonomy and complexity with *Vibrio spp.* (Abbott et al., 2003; Soler et al., 2003; Lamy et al., 2011). It was noted that MicroScan walkaway was also misidentify the *A. schubertii* as *V. damsela* (Soler et al., 2003). The *Aeromonas*

identification requires complex and definitive level such as phenospecies or genomospecies identification (Abbott et al., 2003).

The atypical or inconsistent biochemical results, such as methyl-red, ornithine decarboxylase and gelatin hydrolysis, were clearly detected among *A. schubertii* isolates in this study as well as with reference *A. schubertii* reference strains. It seems the *Aeromonas* family has diversity in phenotype characteristics whether it is clinical or environmental isolation (Carnahan et al., 1991; Abbott et al., 2003). This diversity may occurs because of dissemination of phenotype determinant bearing clones, extrachromosomal element, as well as horizontal and vertical transferring metabolic activities genes (Abbott et al., 2003). For example, putative mobile genetic elements harboring virulence factor of *A. hydrophila* were transferred to their lineage which may increase virulence dramatically (Hossain et al., 2013; Piotrowska and Popowska, 2015). Because of extensive and disseminated clones through horizontal or vertical transfer of hemolytic *Aeromonas* family (i.e. *A. hydrophila* or *A. veronii*), Canadian study found 35 strains of *A. caviae* with unique beta-hemolysin in clinical isolates (Abbott et al., 2003). For other examples, D-xylose fermenting *A. hydrophila* (Okpokwasili, 1991), O/129 sensitive clinical isolates of *A. hydrophila* in Japan (Sugita et al., 1994) and incongruent biochemical test of *A. hydrophila* and *A. salmonicida* in diseased fish (Beaz-Hidalgo et al., 2010). Hence, *Aeromonas* identification by using either conventional biochemical or commercial systems should be identified to the

complex level or more important biochemical profile which is able to discriminate between either *Aeromonas* and *Vibrio* or among *Aeromonas* species itself. which is in this study, *A. schubertii* was able to be distinguished by using MR, VP, amino decarboxylase, esculin hydrolysis, sugar fermentation (mannitol, maltose, mannose, etc.) which is similar to the suggested systems Aerokey II by Carnahan et al. (1991) and Moeller reactions by Abbott et al. (2003). The updated database for identification systems by increasing number strains on the database and improved interpretative criteria was special mention (Carnahan et al., 1991; Abbott et al., 2003; Lamy et al., 2011)

Moreover, PCR assay and identification based on 16S rRNA sequences seemingly offered satisfactory outcome. It showed almost 99.9% similar identity of *A. schubertii* in this study with known *A. schubertii* strains. Furthermore, the phylogenetic analysis based on 16S rRNA and *gyrB* sequences indicated that those *A. schubertii* in this study has close relationship with reference *A. schubertii* strains, especially with those HYL1 strain and ZS20100725 strain. Even though, 16S rRNA sequence has incongruity in several cases, such as lacking on separating closed relations between *A. ichthiosmia* and *A. veronii* as well as *A. simiae* and *A. schubertii*, the relationship of *Aeromonas* family created by derived 16S rRNA had been proven as useful tool (Martinez-Murcia et al., 1992; Collins et al., 1993; Harf-Monteil et al., 2004). Molecular analysis based on housekeeping genes proven as comprehensive

tool for genotypic identification and characterization on *Aeromonas* spp.. it is due to its conserved and distinctive consensus region. However, concatenated housekeeping genes (*gyrB*, *rpo*D, *rpo*B, *dnaJ*, etc.) gives more conserved higher resolution on identification and characterization of *Aeromonas* family (Yanez et al., 2003; Soler et al., 2004; Janda and Abbott, 2010; Martinez-Murcia et al., 2011; Beaz-Hidalgo et al., 2015).

Our finding for measuring the spectra of *A. schubertii* by utilization matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). It seemingly that this study isolates have identical pattern of spectra compared to other *Aeromonas* sp. isolates based on gel stack view. Moreover, principal component analysis (PCA) ad dendrogram constructing were conducted prior to prove whether this study *A. schubertii* isolates were able to be distinguished with other isolates. It was proven identical tree and likelihood of compared the dendrogram created based on MALDI-TOF MS spectra and phylogenetic tree based on housekeeping gene (*gyrB*) sequences (Benagli et al., 2012). Moreover, 2D illustration constructed based on MALDI-TOF MS spectra showed clustered *Aeromonas sp.* which each *Aeromonas* has their own protein mass fingerprint (Pérez-Sancho et al., 2018). Clustered *A. schubertii* in this study were similar to Benagli et al. (2012)'s dendrogram which *A. schubertii* was clustered with *Aeromonas* group 501.

A. schubertii has typical characteristics with *Aeromonas* group 501 (Hickman-Brenner et al., 1988)

The cumulative mortality rate peak was calculated 86% on 21-days post injection (dpi) on high dose. It was higher mortality rate compared to natural outbreak on July 2020 which was approximately 40%. Ren et al. (2019) had compared *A. schubertii* simulation infection with Nile tilapia with intraperitoneal injection, intramuscular injection, per oral administration and bath exposure. It showed it was not more than 80% mortality rates on Intra-peritoneal injection which is the highest mortality rate. Liu and Li (2012) and (Chen et al., 2012) had also reported 80% mortality rate happened in *A. schubertii* challenge in northern and blotched snakehead fish. This is due to intra-peritoneal or other injectable method may not mimic the natural infections (Kim et al., 2011; Ren et al., 2019). Moreover, the immune mechanism or other body mechanism of the fish which using other method (per-oral or immersion) also may have their own way to resist the pathogen before reaching the organs of the host (Kim et al., 2011; Ren et al., 2019). It still remains unknown the route of natural infection of *A. schubertii* (Ren et al., 2019). On the other hand, the median lethal dose (LD50) was formulated by using Reed and Muench (1938)'s formula which was $3.9x10^5$ dose of *A. schubertii*. other experimental challenges of *A. schubertii* were conducted which discovered LD_{50} $6.4x10^6$ and $4.91x10^4$on northern snakehead fish and Nile tilapia, respectively (Chen et al., 2012;

Ren et al., 2019). Mortality rates and LD_{50} may vary between strains of *A schubertii.* it may be due to its virulence factor, pathogenesis, and pathogenicity as well as host size, immune systems and environment (Carraschi et al., 2012; Song et al., 2014; Dias et al., 2016).

The experimental challenge on the isolates was conducted prior to fulfill the Koch's postulates. Our investigations on gross pathology and histopathology analysis of *A. schubertii* infection on striped snakehead fish showed lethargy, skin ulceration, Internal hemorrhage, white nodules showed on the experimental specimens were able to be imitated as similar as natural outbreak happened in July 2020. the experimental challenge of CUVETASC03 to striped snakehead fish showed skin ulceration and hemorrhage apparently were higher observed on 21-dpi than 7-dpi and 14-dpi. It was noted that the skin ulceration and internal hemorrhages were able to be observed in all time of period (7-, 14-, and 21-dpi). skin ulceration and internal organ hemorrhages are the main symptoms of *A. schubertii* infections as similar as those in *A. hydrophila* and *A. veronii* (Zheng, 2013; Rasmussen-Ivey et al., 2016; Dong et al., 2017; Ren et al., 2019; Samayanpaulraj et al., 2019). it had reported that the T3SS of *Aeromonas spp.* was associated with cell-mediated cytotoxin which it may cause tissue damage and destruction of host cell membrane. Their assay reported this secretion systems caused shrinking cells, followed by detaching, loss of cytoplasmic extensions, disorganizing cell monolayer of the HEp-2 cells (Krzymińska

et al., 2012). However, white nodules in this study were significantly spotted in each time periods of observation, yet it was evidently lower observed on 7-dpi than those on 14-dpi and 21-dpi. it also had reported that the white nodules were high occurrence in experimental infection of *A. schubertii* in blotched snakehead fish (Liu and Li, 2012).. White nodules disease may be also caused by any bacteria such as *Francisella* sp. (Lin et al., 2015), *Edwarsiella sp.* (Rousselet et al., 2018), *Mycobacterium* spp. (Madigan et al., 2017; Johansen et al., 2018; Kato et al., 2019), and Nocardia sp. (Wang et al., 2014). Yet, non-bacterial white nodules disease had been reported such as microsporidians (West et al., 2014; Phelps et al., 2015; Picard-Sánchez et al., 2020) and digeneans (Shimazu, 2013). White nodules perhaps are formed from containment of persisting pathogen and lymphocytic cells layered with fibrotic tissue, so that it forms white or yellow lesions with rigid or calcified consistency in internal organs (Noga et al., 1989; Martinot, 2018; Rajme-Manzur et al., 2021). Chen et al. (2012) and Ren et al. (2019) tried to mimic this symptoms in northern snakehead fish and Nile tilapia, but no white nodules were observed. this may be happened due to differences of host immunity response, bacteria serotype and virulence, and environment (Ren et al., 2019).

Furthermore, our finding on experimental challenge of CUVETASC03 to striped snakehead was the necrotic cells (NCs) occurrences was high on 14-dpi and 21-dpi in each organ (liver, kidney, and spleen) and it was lesser occurred on 7-dpi.

Aeromonas spp. had been reported it has T3SS of which causes cytopathic lesions as well as it has Shiga-like toxin which may cause vascular damage and tissue necrosis (Migaki et al., 1975; Palma-Martínez et al., 2016). However, melano-macrophage centers (MMCs) were observed progressively from 7-, 14-, until 21-dpi in each organ. yet, in kidney, it was no significance MMCs observed on 7- and 14-dpi unlike the other organs. It showed also the MMCs is highly found in spleen and kidney. MMCs was highly observed in spleen and kidney (especially head kidney) due to its function as immunological mechanism against infection. Kidney and spleen are hematopoietic organs which play role on immune system mechanism. Moreover, MMCs also rarely occurs in liver and other organs (Steinel and Bolnick, 2017). MMCs are in charged on toxin cleansing, debris scavenging, and phagocytic activity (Herráez and Zapata, 1986; Brattgjerd and Evensen, 1996; Couillard and Hodson, 1996; Davies et al., 2013). Our findings of granulomas in each internal organs were same progressive occurrence start from 7-dpi, 14-dpi until 21-dpi. On the 7-dpi, the granulomas However, it was highly occurred on 14- and 21-dpi in kidney and spleen and both did not show any significance. The granulomas are formed by accumulation infected macrophage which is containment of persistent specific pathogens and other white blood cells (neutrophile, basophil, eosinophil, etc.) as well as fibroblast into heterogeneous compact mass associated with numerous enzymes and cytokines. (James, 2000; Huang et al., 2021; Martínez-Lara et al., 2021; Rajme-Manzur et al., 2021). Tumor necrotic factor alpha (TNF-α), interferon gamma (IFN- γ), interleukins (IL-2, IL-12, IL-8),

pattern recognition receptors (PRRs) such as TLR start the proinflammatory reaction in formation sites to promote monocytes and lymphocytes to the infection sites. Then, $CD4^+$ T cells enter the sites and play as central role in organization of inflammatory cells in sites by promoting more monocytes and lymphocytes and inhibiting the mobility of these inflammatory cells as forming a granuloma. This reaction may extend the process due to the immune systems try to eliminate these evasive pathogens (Green et al., 2013; Pagán and Ramakrishnan, 2018; Rajme-Manzur et al., 2021). *Mycobacterium s*p., *Nocardia* sp., or *Francisella* sp. infections often show granuloma symptoms to the host. It occasionally causes those of *Aeromonas schubertii* nodular symptoms were suspected as those in M*ycobacterium* sp., *Nocardia* sp., or *Francisella* sp. (Liu and Li, 2012).

The gross pathology lesions and histopathology lesions in liver, kidney, and spleen reached the peak in high dose ($1.2x10^8$) of *A. schubertii* challenged in striped head fish. Yet, it was still clearly visible those in lower dose ($1.2x10^3$, 10^4, 10^5, 10^6, and 10^7) rather than those in control group. we speculated that this *A. schubertii* has virulence as high as other *Aeromonas* spp.. however, the report said *A. schubertii* caused higher upregulation of TNF-α, Il-1β, transforming growth factor beta (TGF-β), IL-10 and HSP-70 to the host immune system than those which is infected by *A. hydrophila* (Han et al., 2020; Liu et al., 2021). In addition, it had been reported may cause apoptotic of the host cell by upregulating the “death” receptors signaling

pathways such as TNF-α, TNF-R1, TRAF2, IRAK, CASP8, CASP3 and CASP7. Therefore, this apoptotic cell pathway may be involved in formation of granulomas as those of clinical mycobacterial granulomas. Granulomas contain "core" or bacterial nidus which enlarges and promotes the progressive necrosis or apoptosis to surround tissue (Liu et al., 2021; Martínez-Lara et al., 2021; Rajme-Manzur et al., 2021).

In addition, the white nodules of gross pathology lesions and granulomas of histopathology lesions in liver, kidney, and spleen were highly expressed in 14- and 21-dpi and the peak mortality of *A. schubertii* infection in recent study showed 86% on 21-dpi. we speculated that *A. schubertii* also may prolong the surviving host from the severity of granuloma pathologies. Ren et al. (2020) had reported that *A. schubertii* is able to upregulate the IL-10 and TGF-β in host immune systems**.** the IL-10 and TGF-β are important anti-inflammatory cytokines which counteract to the proinflammatory cytokines (Kondera, 2011). Thus, this counteracts reaction may play role on surviving host cell from infectious granuloma pathologies and may carry the pathogen for extending period of infection (Rajme-Manzur et al., 2021).

Our finding showed highly strong positive correlation between white nodules and granulomas in kidney and spleen. meanwhile, it showed weak positive association in liver white nodules and granuloma. Granulomas may visible as white nodules in organs surface. Granuloma caused by *Nocardia* sp., *Francisella* sp. and *Mycobacterium* sp. are often manifested as white or grey nodules in the organ

surface (liver, kidney and spleen) (Wang et al., 2007; Wang et al., 2014; Lin et al., 2015; Johansen et al., 2018; Martínez-Lara et al., 2021). Granuloma may form white, grey, or yellow lesions in organs surface due to its formation of “core” covered with fibrotic tissue and its favor consequently to encapsulate and eliminate the persistent pathogens and damaged tissue (James, 2000; Martinot, 2018; Martínez-Lara et al., 2021; Rajme-Manzur et al., 2021). the *A. schubertii* is able to upregulate the TGF-β in host immune system which had been reported that it is also highly associated with collagen production in active granuloma which may cause fibrotic environment in granuloma formation sites (DiFazio et al., 2016; Ren et al., 2020). The Granuloma formation site may be easily correlated with robust and complex inflammatory reactions involving cytokines, fibroblast, macrophages, and any other leucocytes which are contained in hematopoietic organs, such as spleen and kidney (Plouffe et al., 2005; Aoki, 2008; Castro et al., 2019).

5.2 Conclusions.

Characterizations of *A. schubertii* by utilization of biochemical assay, PCR and DNA sequencing based on 16S rRNA and *gyrB* and MALDI-TOF MS provided typical biochemical characteristics, DNA fingerprint and protein mass fingerprint of *A. schubertii* which are able to be used as an establishment of *A. schubertii* identification method to other bacteria or even other *Aeromonas* spp.. Reconstruction of *A. schubertii* outbreak in July 2020 in Nakhon Pathom province, Thailand by experimental challenge acquired identical and precise symptoms on striped snakehead fish (*C. striata*) which were skin ulceration, internal organ hemorrhage, white nodules, and manifestation of granuloma, melano-macrophage centers (MMCs) and necrotic cells in liver, kidney and spleen histopathology observation. In addition, it was discovered manifestation of white nodules in kidney and spleen of gross pathology lesions were strongly correlated with granuloma in kidney and spleen of histopathology lesions.

5.3 Further Investigations.

Present study provides information of biochemical characteristics, DNA fingerprint based on 16S rRNA and *gyrB*, protein mass fingerprints as well as pathogenicity of *A. schubertii* in striped snakehead fish (*C. striata*). Thus, further investigation should be more implemented on numerous strains of *A. schubertii* whether it is from discrete area or host. It may lead into clear characteristics and pathogenicity of *A. schubertii*. In addition, deep explorations on pathogenicity and pathogenesis of *A. schubertii* by utilization of new technique of combination histopathology, DNA hybridization and immunocyto-histo chemistry may discover precise and understandable pathogenesis of *A. schubertii*.

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APPENDIX A Media preparation

A.1. Hemolysin Test

Principle :

Hemolysin test uses tryptic soy agar (TSA) supplemented with 5% sheep blood. TSA is used as highly nutritious medium for the microorganism. Sheep blood (defibrinated) is used to determine hemolytic activity of the microorganism.

Result: α-hemolysin : incomplete translucent zone around bacteria growth

β-hemolysin : Clear zone around bacteria growth

Negative : No clear zone around bacteria growth

Preparation :

Tryptic soy agar BD BBL™..... 40 g

Composition : Pancreatic Digest of Casein..... 14.5 g

Papaic Digest of Soybean Meal..... 5.0 g

Sodium Chloride..... 5.0 g

Agar..... 14.0 g

Growth Factors..... 1.5 g

Distilled water .....950 ml

Sheep blood (defibrinated) .....50 ml

Mix the ingredients and autoclave at 121oC except the defibrinated sheep blood. Let it cool down at ~47 oC. aseptically add defibrinated sheep blood 50 ml. mix gently and dispense as required. Transfer a single bacteria colony or suspended bacteria onto the agar. Then streak gently by using inoculation loop. Incubate at 28oC for 24 hr.

A.2. Motility Test

Principle :

Motility test is designed to determine the motility of the organism. which is some bacteria has organelles such as flagella, cilia, fimbriae to make them glide, swim, or

swarm. Motile organism is demonstrated diffuse spreading growth in semi solid agar. While, non motile organism is presented localized growth in semi solid agar.

Result:

Motile (+) : Diffused pink color growth

Non-motile (-) : Localized pink color growth

Preparation :

Tryptose ..... 10.0 g

NaCl ..... 5.0 g

Agar..... 5.0 g

Distilled water..... 1000 ml

Triphenyl tetrazolium chloride (TTC) ..... 0.05 g

Mix the ingredients and autoclave at 121oC. add TTC to the medium. Dispense into 10ml glass tube. Aseptically transfer a single colony of bacteria into the tube by using straight stab needle. Incubate at 28oC for 24 hr.

A.3. Gelatin Hydrolysis.

Principle :

Gelatin Hydrolysis test is designed to detect the ability of the organism to produce gelatinase which liquify the gelatin. Gelatinase degrades gelatin into polypeptides. Then, it converts into amino acids.

Result:

Positive (+) : Partial or total liquified medium

Negative (-) : Complete solid medium

Preparation :

Tryptose..... 20.0 g

Beef extract..... 3.0 g

Gelatin..... 10.0 g

Manganese sulfate..... .....0.01g

Agar..... 15.0 g

Distilled water..... 1000 ml

Autoclave at 121oC. Dispense into tube. Tubes is cooled in an upright position. Aseptically transfer a single colony of bacteria into the tube by using straight stab needle. Add 4-5 drops of a 24-hr broth medium. Incubate at 28oC for 24 hr.

A.4. Lipid Hydrolysis

Principle :

Lipid hydrolysis test is designed to determine ability of the organism producing lipase enzyme to hydrolyse lipid. Lipid will be degraded by the organism into triglycerides by cleaving the ester bonds of the lipid molecule.

Result:

Positive (+) : Formation of a clear zone around bacteria growth

Negative (-) : No clear zone around growth

Preparation :

Peptic digest animal tissue..... 5.0 g

Yeast extract..... 3.0 g

Agar..... 15.0 g

Distilled water..... 1000 ml

Autoclave at 121oC. Dispense into petri disks. Aseptically transfer a single colony of bacteria into the tube by using inoculation loop with single line streaking. Incubate at 28oC for 24 hr.

A.5. Casein Hydrolysis

Principle :

Casein hydrolysis is designed to determine the ability of the organism to degrade casein protein from skim milk. The organism uses protease enzyme (caseinase) to cleave the peptide bond CO-NH of the casein by introducing water molecule (hydrolyse) into peptones, polypeptides, dipeptides or amino acids.

Result:

Positive (+) : Formation of a clear zone around bacteria growth

Negative (-) : No clear zone around growth

Preparation :

Skim milk powder..... 28.0 g

Tryptone..... 5.0 g

Yeast extract..... 3.0 g

Dextrose ..... 1.0 g

Agar..... 15.0 g

Distilled water..... 1000 ml

Dilute all ingredient into 1000 ml distilled water except skim milk powder. Autoclave at 121oC. add skim milk powder into the medium when it is 48 – 50oC. Dispense into petri disks. Wait until it becomes solid and dry. Aseptically transfer a single colony of bacteria into the tube by using inoculation loop with single line streaking. Incubate at 28oC for 24 hr.

A.6. Lecithinase Test

Principle :

Lecithinase test is designed to determine the ability of the organism to degrade lecithin from egg yolk. Lecithin is composed by phospholipids linked to choline which is degraded by lecithinase of the organism and forms precipitation on the medium.

Result:

Positive (+) : Formation of precipitation zone around bacteria growth

Negative (-) : No precipitation zone around growth

Preparation :

Yeast extract..... 5.0 g

NaCl..... 5.0 g

Tryptone .... 5.0 g

Proteose peptone ..... 20.0 g

Egg yolk.....100.0 ml

Agar..... 20.0 g

Distilled water..... 900 ml

Two fresh eggs are brushed and dipped into 70% ethanol for 1 hr. collect egg yolk aseptically. Add saline water 0.85% equal volume as the egg yolk volume. Mix all of

the ingredient except egg yolk emulsion and autoclave at 121oC. add egg yolk emulsion into the medium when it is 48 – 50oC. Dispense into petri disks. Wait until it becomes solid and dry. Aseptically transfer a single colony of bacteria into the tube by using inoculation loop with single line streaking. Incubate at 28oC for 24 hr.

A.7. Oxidative-Fermentative Test

Principle :

Oxidative-fermentative (OF) test is designed to determine the ability of the organism to metabolize glucose (carbohydrate) by fermentation (anaerobic) or oxidative (aerobic). During the organism metabolizing the glucose, it converts pyruvate into variety mixed acids. Accumulation of acids turn the bromthymol blue indicator from green to yellow.

Result :

No liquid paraffin added (aerobic) Liquid paraffin added (anaerobic)

Interpretation

Yellow (surface) Green (no change) Oxidative (obligate aerobic)

Yellow (bottom) Yellow Fermentative (obligate anaerobic)

Yellow Yellow Fermentative (facultative anaerobic)

Green (no change) Green (no change) Non saccharolytic organism

Preparation :

Peptone..... 2.0 g

NaCl ..... 5.0 g

K2PO4 ..... 0.3 g

Bromthymol blue ..... 0.03 g

Agar..... 5.0 g

Distilled water..... 1000 ml

Glucose ..... 10.0 g

Mix the ingredients and autoclave at 121oC. Let it cool down at ~47oC and add the glucose. Dispense into 10ml glass tube. Aseptically transfer a single colony of bacteria into each tube by using straight stab needle. Add 4-5 drops of liquid paraffin

for anaerobic condition and no liquid paraffin drop on aerobic condition. Incubate at 28oC for 24 hr.

A.8. Esculin Hydrolysis Test

Principle :

Esculin hydrolysis test is designed to determine gram negative bacteria to hydrolyze esculin into esculetin. Esculetin reacts with Fe3+ ions and forms dark brown to black precipitation. Bile substance makes gram-positive bacteria unable to grow.

Result:

Positive (+) : Blackened zone on bacteria growth

Negative (-) : No blackened zone on bacteria growth

Preparation :

Bile esculin agar BD BBL™..... 40 g

Composition : Pancreatic Digest of Gelatin..... 5.0 g

Beef Extract ..... 3.0 g

Ox gall ..... 20.0 g

Ferric Citrate..... 0.5 g

Esculin ..... 1.0 g

Agar..... 14.0 g

Distilled water .....1000 ml

Mix the ingredients and autoclave at 121oC. dispense into petri disks. Let it become solid and dry. Aseptically transfer a single bacteria colony with single streak onto the medium by using inoculation loop. Incubate at 28oC for 24 hr.

A.9. Indole Test

Principle :

Indole test is designed to determine the ability of the organism which is able to undergo hydrolysis and deamination to tryptophan by using tryptophanase. Tryptophanase remove amine group of tryptophan which produce indole, pyruvic acid and ammonium. When indole reacts with Kovac reagent (HCl and p-dimethylaminobenzaldehyde in amyl alcohol). It turns yellow color into cherry red.

Result:

Positive (+) : Formation of red ring on medium surface

Negative (-) : Formation of yellow ring on medium surface

Preparation :

Tryptone..... 5.0 g

NaCl ..... 15.0 g

Distilled water..... 1000 ml

Kovac's reagent ..... 4-5 drops

Mix the ingredients and autoclave at 121oC. Dispense into 5 ml glass tube. Let it cool down. Aseptically transfer a single colony of bacteria into the tube by using inoculation loop. Incubate at 28oC for 24 hr. add Kovac's reagent 4-5 drops. Observe the color changing.

A.10. Voges Proskauer (VP) And Methyl-Red (MR) Test

Principle :

VP test is designed to determine the organism is able to produce acetylmethyl carbinol from glucose fermentation. If presence of acetylmethyl carbinol reacts with α-naphthol and converted into diacetyl. Diacetyl and quinidine compounds react with strong alkali (KOH), will form pinkish red color.

MR test is designed to determine the ability of the organism to produce acid during glucose fermentation and maintain the condition such as lactic acid bacteria which can maintain acidic environment. The methyl red will change into yellow if reacts with pH 6.2.

Result:

Positive (+) VP : Formation of pink red color in broth

Negative (-) VP : Formation of yellow color in broth

Positive (+) MR: Formation of red color in broth

Negative (-) MR: Formation of yellow color in broth

Preparation :

Buffered peptone..... 7.0 g

K2PO4 ..... 15.0 g

Dextrose ..... 5.0 g

Distilled water..... 1000 ml

Voges Proskauer reagents

- Barrit's reagent A : **α**-naphthol in absolute alcohol..... 5% (w/v)

- Barrit's reagent B : KOH ..... 40% (w/v)

Methyl red solution :

Composition : Methyl red ..... 0.1 g

Absolute ethanol 300ml

Deionized water ..... 200 ml

Mix the ingredients and autoclave at 121oC. Dispense into 5 ml glass tube. Let it cool down. Aseptically transfer a single colony of each isolate bacteria into the two different tube by using inoculation loop. Incubate at 28oC for 24 hr.

For VP test : add 4-6 drops of Barrit's reagent A and mix well. Then add 2 drops of Barrit;s reagent B. Observe the color changing.

For MR test : add 4-6 drops of methyl red solution and mix well. Observe the color changing.

A.11. Amino Acid Decarboxylations (Arginine, Lysine and Ornithine)

Principle :

Amino acid decarboxylations are designed to determine the organism produce decarboxylases which is able to react with carboxyl group of amino acids and forms alkaline-reacting amines. Non-decarboxylase-produced organism will ferment the dextrose and change into acidic environment of the broth. And it reacts with pH indicator (bromo cresol purple) and resulting color change from purple to yellow. If decarboxylase-produced organism will maintain or increase the pH because of alkaline amine accumulation.

Result:

Positive (+) : No changing color or purple color in broth

Negative (-) : Forming yellow color in broth

Preparation :

Peptic digest of animal tissue..... 5.0 g

Yeast extract ..... 3.0 g

Dextrose ..... 1.0 g

Bromo cresol purple ..... 0.02 g

Distilled water..... 1000 ml

L-lysine hydrochloride ..... 0.5% final concentration

L-ornithine hydrochloride ..... 0.5% final concentration

L arginine hydrochloride ..... 0.5% final concentration

Mix the ingredients and autoclave at 121oC. let it cool down at 47oC. add separately amino acids (L-Lysine hydrochloride, L-ornithine hydrochloride and L-arginine hydrochloride) to final concentration of 0.5%. Dispense into 5 ml glass tube. Aseptically transfer a single colony of each bacteria isolates into each of amino acid tubes by using inoculation loop. Add 4-5 drops of liquid paraffin. Incubate at 28oC for 24 hr. Observe the color changing.

A.12. Sugar Fermentation Test (Mannitol, Dextrose, Arabinose, Sucrose and Lactose)

Principle :

Sugar fermentation test is designed to determine the ability of the organism to metabolize carbohydrates (Mannitol, Dextrose, Arabinose, Sucrose and Lactose) by fermentation. organism will ferment the carbohydrates into pyruvic acid and change into acidic environment in the broth. It will react with pH indicator (bromo cresol purple) and resulting color change from purple to yellow.

Result :

Positive (+) : Yellow color

Negative (-) : no change color or purple color

Gas formation positive (+) : oxygen bubble inside Durham tube

Gas formation negative (-) : no oxygen bubble inside Durham tube

Preparation :

Proteose peptone ..... 10.0 g

Beef extract ..... 1.0 g

NaCl ..... 5.0 g

Bromocresol purple ..... 0.02 g

Distilled water..... 1000 ml

Mix the ingredients and autoclave at 121oC. let it cool down at 47oC. add separately carbohydrates (Mannitol, Dextrose, Arabinose, Sucrose and Lactose) to final concentration of 0.5%. Dispense into 5 ml glass tube with Durham tube inside. Aseptically transfer a single colony of each bacteria isolates into each of carbohydrates tubes by using inoculation loop. Incubate at 28oC for 24 hr. Observe the color changing.

APPENDIX B Method and technique

B.1. Gram Staining

Principle :

Bacterial peptidoglycan in cell membrane determines the bacteria gram type. Gram positive bacteria has thicker peptidoglycans than gram negative bacteria.

Result Interpretation :

Gram positive : purple color organism

Gram negative : red color organism

Reagents preparation :

- Crystal violet
- Gram's Iodine Solution
- Acetone/Ethanol (50:50 v/v)
- Safranin

Method :

- Aseptically transfer suspended culture to the microscope or single bacterial colony from petri dish into a drop of water on the microscope slide. Then smear gently and let it dry.
- add drops of crystal violet and wait for 10-60 s. then rinse with water
- add drops of Iodine solution and wait for 10-60 s. then rinse with water
- rinse with Acetone/Ethanol gently for 5 s. then rinse with water.
- add drops of Safranin and wait for 10-60 s. then rinse with water. And then let it dry
- the specimen is ready to be observed under light microscope. Use immersion oil if examined under 100x magnification.

B.2. Oxidase Test

Principle :

Oxidase test is designed to detect the presence of cytochrome C oxidase by observing oxidizing activity to the reagent and developing dark purple colorization on the filter disk.

Result Interpretation :

Positive : dark purple colorization

Negative : No color change or colorless

Reagents preparation :

- Reagent tetramethyl-p-phenylenediamine dihydrochloride
- Filter paper Whatman no. 1

Method :

- Add a drop of Reagent tetramethyl-p-phenylenediamine dihydrochloride onto filter paper
- Aseptically transfer single bacterial colony from petri disk on filter paper. Then smear gently.
- Observe for color changing within 10 s.

B.3. Catalase Test

Principle :

Catalase test is designed to detect the presence of Catalase enzyme produced the organism by observing decomposing hydrogen peroxide and releasing water and oxygen.

Result Interpretation :

Positive : Oxygen bubble formation

Negative : No oxygen bubble formation

Reagents preparation :

- Hydrogen Peroxide (H_2O_2)
- Microscope slide

Method :

- Add a drop of hydrogen peroxide onto microscope slide
- Aseptically transfer single bacterial colony from petri disk on the hydrogen peroxide.
- Observe for oxygen bubble formation within 10 s.

B.4. Hematoxylin and Eosin Staining

Principle :

Hematoxylin and Eosin staining is designed to stain and recognize the different types of tissues. Hematoxylin has a deep blue purple color which will stain nucleic acids or nucleus of the cells. Otherwise, eosin is red color dye which will stain non-specific protein in cytoplasm and extracellular matrix.

Reagents preparation :

- Xylene
- 70, 95 and 100% ethanol
- wax (paraplast X-tra)
- Paraffin wax
- 0.3% acid alcohol
- scott's tap water (magnesium sulfate and sodium bicarbonate)
- Alum hematoxylin
- Eosin Y

Tissue preparation and dehydration :

- trim the tissue into 20 mm x 30 mm and 3 mm thickness.
- mount the tissue into the cassette with label
- dip into 70% ethanol for 1 hr
- dip into 95% ethanol for 1 hr
- dip into first absolute ethanol for 1 hr
- dip into second ethanol for 1.5 hr
- dip into third absolute ethanol for 1.5 hr
- dip into fourth absolute ethanol for 2 hr.
- dip into first clearing agent (xylene) for 1 hr
- dip into second clearing agent (xylene) for 1 hr.
- dip first wax (paraplast x-tra) at 58oC for 1 hr
- dip second wax (paraplast x-tra) at 58oC for 1 hr

Embedding and microtome :

- open cassette and discard the lid
- pour small amount of molten paraffin wax into the mold
- using warm forceps, transfer the tissue into the mold
- transfer the mold to cold plate and gently press the tissue flat.

- as if the tissue is in desired orientation, label and press firmly
- add molten paraffin into the mold and make sure that is enough to caver the face of plastic cassette.
- let it solid within 30 min. if there is any crack of the paraffin block or the tissue is aligned undesirable, melt it and start over
- place the embedded tissue into microtome machine as the blade facing desired position of the tissue
- set the initial cut 10 μm to trim paraffin ribbon
- as if it is not ribboning, set into 5 μm and cut the block 4 times.
- pick up the cut section with forceps or fine paint brush
- float them in the surface of 37oC water bath.
- pick up the floated section with clear glass slide then put it the 65oC to melt the paraffin.

Hematoxylin and eosin staining :

Dewaxing : - dip mounted tissue in slide into xylene for 2 min two times.
- dip the slide into 95% ethanol for 2 min two times.
- dip the slide into 70% ethanol for 2 min one time.
- wash with water for 2 min.

Hematoxylin and eosin staining : - stain the slide with hematoxylin for 5 min
- rinse with water gently 1-5 min until the sections turn into blue
- rinse with 0.3% acid alcohol gently for 5 s
- wash in tap water 1-5 min to remove the acid alcohol.
- exposed the slide to bluing solution (scott's tap water)
- rinse in tap water 1-5 min gently to remove bluing solution
- stain with eosin Y for 10 min
- wash in tap water 1-5 min

Dehydrate, clear and mount : - rinse in alcohol solution 70% and 95%
- clear with the clearant (xylene)
- mount the cover slide into the section
- and seal with acrylic resin or plasticizers (clear nail polish)

B.5. Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI TOF MS)

Principle :

MALDI TOF MS is designed to identify the bacteria characteristic mass spectral fingerprint and compared with large library database of mass spectra. MALDI TOF uses pulsed UV light assisted with crystallization of matrix to vaporize the biopolymer of the sample and accelerate within the vacuum chamber. The biopolymer will be detected by detector and presented as mass spectra.

Reagents preparation :

- CHCA (α-cyanno-4-hydroxicinnamic acid) matrix solution dissolved in acetonitrile 50% and 2.5% trifluoroacetic acid
- formic acid 70%
- deionized water
- ethanol
- acetonitrile

Procedure :

Direct mount :

- pick up a single colony of cultured bacteria by using wooden tooth pick or steel needle.
- smear onto a MALDI TOF target plate.
- overlay with 1 µl formic acid and let it dry.
- add 1-2 ml of matrix (CHCA) and let it dry
- place target plate in plate chamber of MALDI TOF MS.

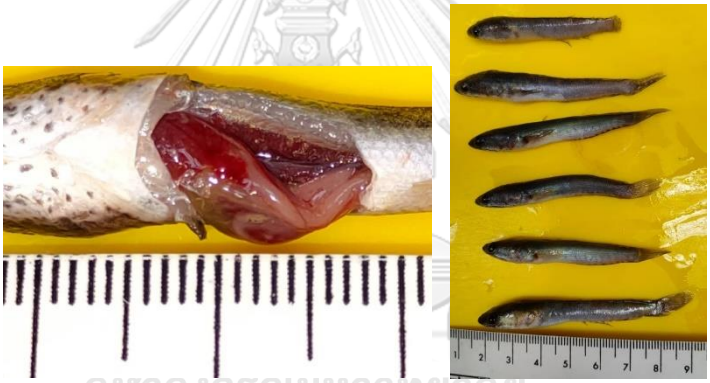
Extraction method :

- transfer a single colony or 5-10 mg of bacteria into an eppendrof tube with 300 µl of deionized water. Mix it well by using vortex
- add 900 µl of ethanol (EtOH) and mix thoroughly.
- centrifuge at ≥ 13.000 rpm for 2 min. remove supernatant
- centrifuge again and remove supernatant without disturbing the pellet. Let it dry.
- add 1-80 µl of formic acid and mix well

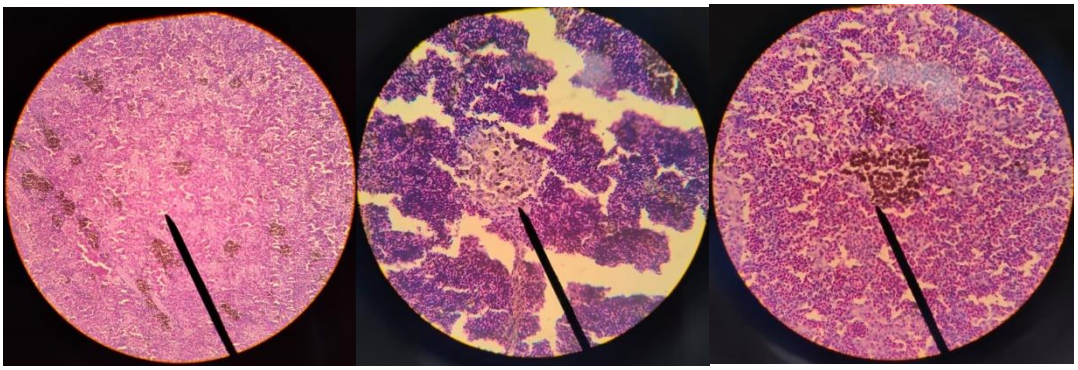
- add 1-80 µl of acetonitrile as same as formic acid volume which has been added.
- centrifuge at ≥ 13.000 rpm for 2 min.
- collect 1 µl of the supernatant and transfer into target plate of MALDI TOF. Let it dry.
- add 1 µl matrix solution (CHCA) and let it dry
- transfer target plate into target chamber of MALDI TOF MS.

APPENDIX C Figures and tables

First isolation of *A. schubertii* from natural infected striped snakehead fish organs in TSA supplemented 5% sheep blood



Striped nakehead fish fingerlings appearance and gross pathology of natural infection



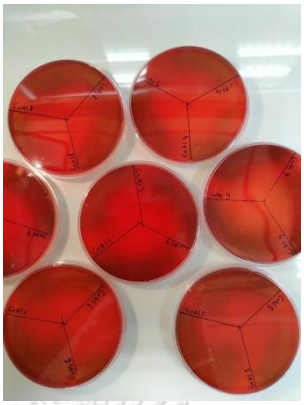
Histology view of natural infected striped snakehead fish organs

Table of details of six isolates of *A. schubertii* isolated from natural infected striped snakehead fish.

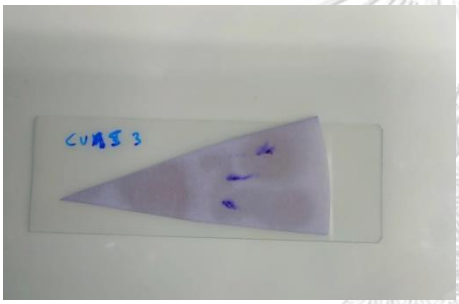
Fish	Gross lesion			Histopathology lesions			Bacteria isolated	internal organ isolated	isolate name
	White nodules	Skin ulceration	Hemorrhage	granuloma	Melanomacrophage center	Necrotic tissue			
Fish 1	√	√	-	√	√	√	*A. schubertii*	Liver, kidney, spleen, and brain	CUVETASC03
Fish 2	-	√	√	√	√	√	*A. schubertii , A. veronii*	Liver, kidney, spleen, and brain	CUVETASC04
Fish 3	√	-	√	√	√	√	*A. schubertii*	Liver, kidney, spleen, and brain	CUVETASC05
Fish 4	√	√	√	√	√	√	*A. schubertii , A. veronii*	Liver, kidney, spleen, and brain	CUVETASC06
Fish 5	-	√	√	√	√	√	*A. schubertii*	Liver, kidney, spleen, and brain	CUVETASC07
Fish 6	√	√	√	√	√	√	*A. schubertii , A. veronii*	Liver, kidney, spleen, and brain	CUVETASC08

Tables of initial Identification of Natural Infection *A. schubertii* by Utilization of Biochemical Assay and MALDI ToF MS.

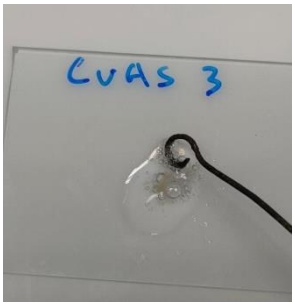
Lot	Fish No.	Organ	Isolate No.	Colony	Gram Shape	-moly	xidas	atala	Motil	ndol	O/F	MR	VP	n hy	rginin	rnith	Lysin	MALDI-TOF (First Best Match)	MALDI-TOF (Second Best Match)
16	1	Internal	1	Spot cream	Short rod (-)	ß	+	+	-	-	+/+	-	-	-	-	-	-	*A.scubertii (2.10)*	No Organism Identification Possible
		Brain	2	Small cream	Short rod (-)	ß	+	+	+	-	+/+	-	-	-	-	-	-	*A.scubertii (2.11)*	No Organism Identification Possible
	2	Internal	3	Big Cream	Short rod (-)	ß	+	-	+	+	+/+	-	-	-	-	-	+	*V.albensis (1.91)*	*V.albensis (1.83)*
		Brain	4	Big Cream	Short rod (-)	α	+	+	+	-	+/+	-	-	-	-	-	-	*A.schubertii (2.03)*	No Organism Identification Possible
	3	Internal	5	Big Orange	Rod (-)	α	+	+	+	-	-/-	-	-	-	+	+	-	No Organism Identification Possible	No Organism Identification Possible
		Brain	24	Big orange	Short rod (-)	-	+	+	+	-	-/-	-	-	-	+	-	-	*Shewanella putrefaciens (1.93)*	*Shewanella putrefaciens (1.87)*
	4	Internal	6	Big Cream	Short rod (-)	ß	+	+	+	+	+/+	-	-	-	-	+	+	*V.albensis (1.84)*	*V.albensis (1.80)*
	5	Internal	7	Big Cream	Short rod (-)	ß	+	+	+	-	+/+	-	-	-	-	+	+	No Organism Identification Possible	No Organism Identification Possible
17	1	Internal	8	Small Cream	Short rod (-)	α	+	+	-	-	-/+	-	-	-	-	-	-	*A.schubertii (2.08)*	*A.schubertii (1.92)*
		Brain	9	Small Cream	Short rod (-)	α	+	+	-	-	+/+	-	-	-	-	-	-	*A.schubertii (2.28)*	No Organism Identification Possible
	2	Internal	10	Big Cream	Short rod (-)	ß	+	+	+	-	+/+	-	-	-	-	-	-	*A.veronii (2.27)*	*A. ichtiosmia (2.18)*
		Brain	11	Small Cream	Short rod (-)	ß	+	+	+	-	+/+	-	-	-	-	-	+	*A.schubertii (2.03)*	No Organism Identification Possible
	3	Internal	12	Big Orange	Rod (-)	-	+	+	+	-	-/-	-	-	-	+	+	-	*Shewanella putrefaciens (1.90)*	*Shewanella putrefaciens (1.85)*
		Brain	13	Big Cream	Short rod (-)	α	+	+	+	-	-/-	-	-	-	+	+	-	*Shewanella putrefaciens (1.94)*	*Shewanella putrefaciens (1.87)*
	4	Internal	14	Spot Cream	Rod (-)	ß	+	+	+	-	-/+	-	-	-	-	-	-	*A.veronii (2.14)*	*A.veronii (2.10)*
	5	Internal	16	Spot Cream	Short rod (-)	ß	+	+	+	-	+/+	-	-	-	-	-	-	*V.albensis (1.91)*	*V.albensis (1.87)*
18	1	Internal	17	Spot Cream	Short rod (-)	α	+	+	-	-	+/+	-	-	-	-	-	-	*A.schubertii (2.23)*	No Organism Identification Possible
		Brain	18	Small cream	Short rod (-)	α	+	+	+	-	+/+	-	-	-	-	-	-	*A.schubertii (2.11)*	No Organism Identification Possible
	2	Internal	19	Big Cream	Coccobacil (-)	α	+	+	+	-	+/+	-	-	-	-	-	-	*A.veronii (2.23)*	*A.veronii (2.17)*
		Brain	20	Small cream	Short rod (-)	-	-	+	-	-	+/+	-	-	+	-	-	-	*A.schubertii (2.08)*	No Organism Identification Possible
	3	Internal	21	Small cream	Big rod (-)	-	+	-	-	-	-/-	-	-	-	+	+	+	No Organism Identification Possible	No Organism Identification Possible
		Brain	22	Big orange	Short rod (-)	-	+	+	+	-	-/-	-	-	-	+	+	-	No Organism Identification Possible	No Organism Identification Possible
	4	Internal	15	Small Cream	Short rod (-)	ß	+	+	+	+	-/+	-	-	-	-	+	+	*V.albensis (1.81)*	No Organism Identification Possible
	5	Internal	23	Small cream	Short rod (-)	ß	+	+	+	-	+/+	+	-	-	-	+	-	*V.albensis (1.85)*	No Organism Identification Possible



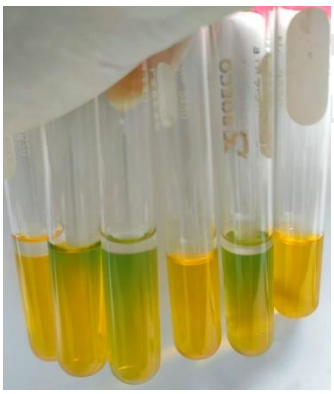
The *A. schubertii* growth on TSA supplemented with 5% sheep



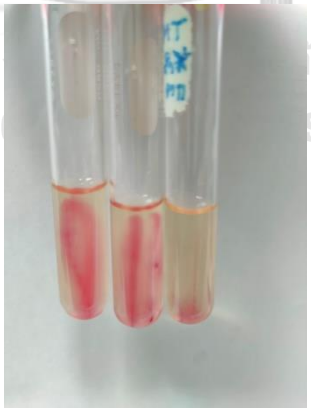
Oxidase test



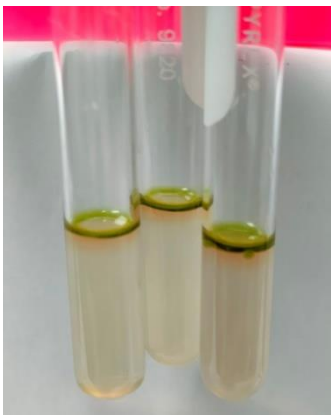
Catalase test



OF test

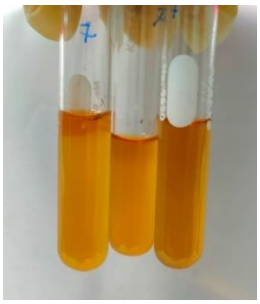


Motility test



Indole production test

Methyl Red test

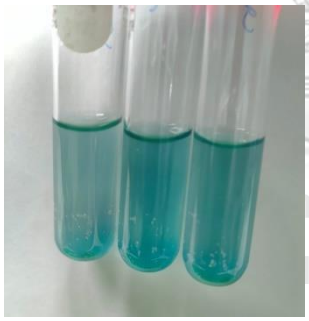


Voges Proskauer test

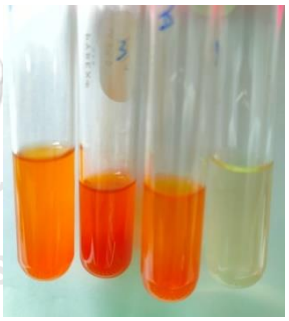
Lysine decarboxylase test

Arginine deaminase test

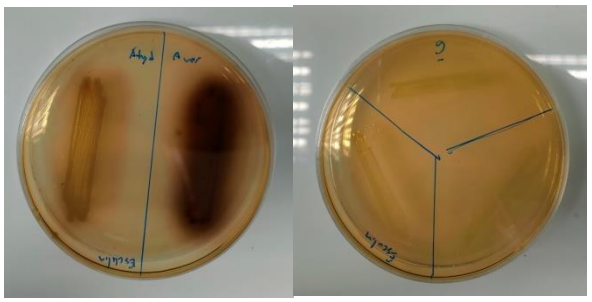
Ornithine decarboxylase test



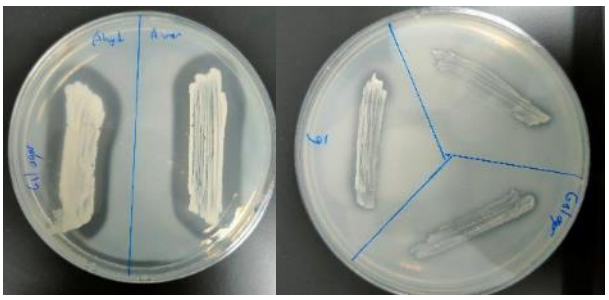
Gluconate test



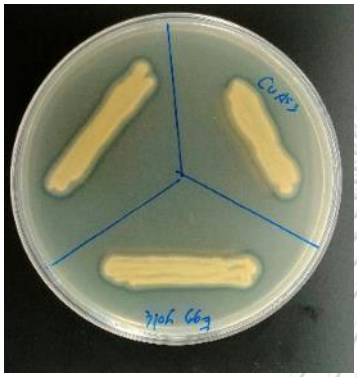
Nitrate reduction test



Esculin hydrolysis test

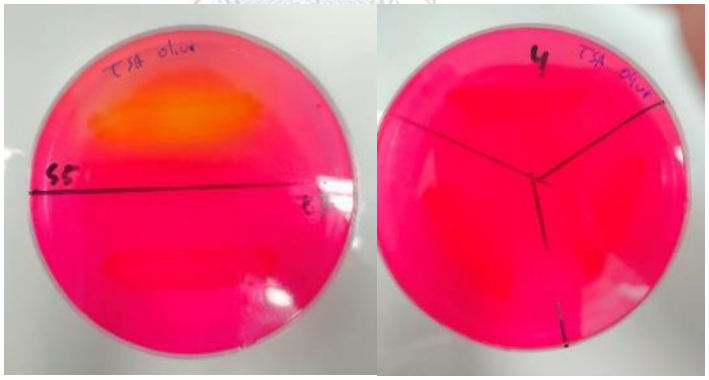


Gelatin hydrolysis test

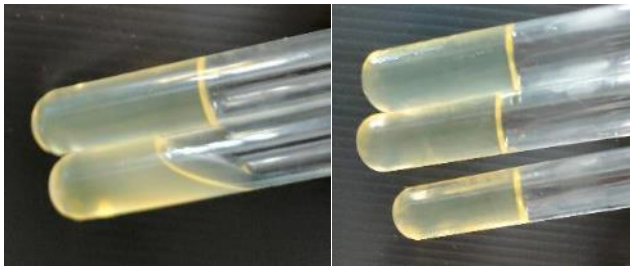


Lecithinase test

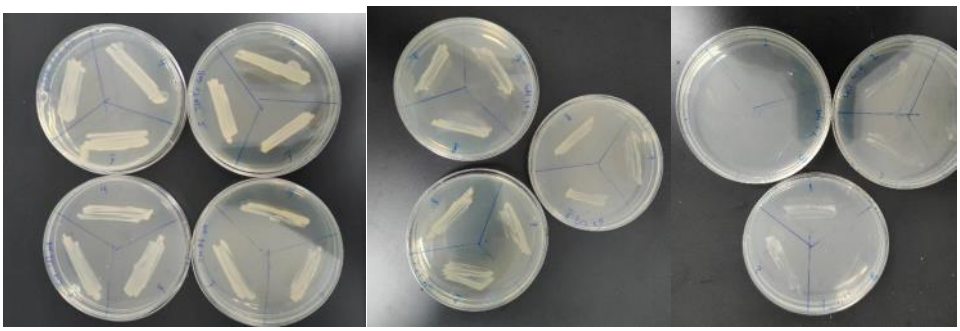
Casein test



Glycerol Fermentation test



Gelatin liquefaction test



The *A. schubertii* in TSA supplemented 0.5-5.0% NaCl

Table of conventional biochemical assay results of *A. schubertii* isolates of this study.

Conventional Biochemical Assay	*Aeromonas schubertii* in this study (CUVETASC)						
	03	04	05	06	07	08	(%)
Gram stain	-	-	-	-	-	-	-
Shape	SR	SR	SR	SR	SR	SR	SR
Oxidase	+	+	+	+	+	+	100
Catalase	+	+	+	+	+	+	100
Hemolysin	+	+	+	+	+	+	100
Motility	+	+	+	+	+	+	100
Oxidative/Fermentative	F	F	F	F	F	F	F
Indole	-	-	-	-	-	-	0
Methyl Red	+	+	+	+	+	+	83.33
Voges Proskauer	-	-	-	-	-	-	100
Arginine deaminase	+	+	+	+	+	+	100
Lysin decarboxylase	+	+	+	+	+	+	16.67
Ornithine decarboxylase	-	-	-	-	+	-	0
Esculine hydrolysis	-	-	-	-	-	-	100
Nitrate reduction	+	+	+	+	+	+	0
Gluconate	-	-	-	-	-	-	100
Maltose	+	+	+	+	+	+	0
Arabinose	-	-	-	-	-	-	0
Sucrose	-	-	-	-	-	-	0
Lactose	-	-	-	-	-	-	100
Galactose	+	+	+	+	+	+	100

Conventional Biochemical Assay	*Aeromonas schubertii* in this study (CUVETASC)						
	03	04	05	06	07	08	(%)
Casein	+	+	+	+	+	+	100
Gelatin hydrolisis	-	-	-	-	-	-	0
Lecithinase	+	+	+	+	+	+	100
Olive oil	-	-	-	-	-	-	0
Glycerol	-	-	-	-	-	-	0
Growth in nutrient plus							
0.5% NaCl	+	+	+	+	+	+	100
1% NaCL	+	+	+	+	+	+	100
1.5% NaCl	+	+	+	+	+	+	100
2% NaCl	+	+	+	+	+	+	100
2.5% NaCl	+	+	+	+	+	+	100
3% NaCl	+	+	+	+	+	+	100
3.5% NaCl	+	+	+	+	+	+	100
4% NaCl	-	-	-	-	-	-	0
4.5% NaCl	-	-	-	-	-	-	0
5% NaCl	-	-	-	-	-	-	0

"SR": short rod, "F": fermentative.

Table of biochemical profile result of *A. schubertii* isolates by using Vitek-2 compact® GN card.

		CUVETASC						
Vitek 2 compact® biochemical test		03	04	05	06	07	08	(%)
APPA	Ala-Phe-Pro-Arylamidase	-	-	-	-	-	-	0
ADO	Adonitol	-	-	-	-	-	-	0
PyrA	L-Pyrrolydonyl-Arylamidase	-	-	-	-	-	-	0
IARL	L-Arabitol	-	-	-	-	-	-	0
dCEL	D-Cellobiose	-	-	-	-	-	-	0
BGAL	Beta-Galactosidase	-	-	-	-	-	-	0
H2S	H_2S production	-	-	-	-	-	-	0
BNAG	Beta-N-Acetyl Glucosaminidase	(-)	+	+	+	+	+	83.33
AGLTp	Glutamyl Arylamidase pNA	-	-	-	-	-	-	0
dGLU	D-Glucose	+	+	+	+	+	+	100
GGT	Gamma-Glutamyl-Transferase	-	-	-	-	-	-	0
OFF	Fermentation/Glucose	+	+	+	+	+	+	100
BGLU	Beta-Glucosidase	-	-	-	-	-	-	0
dMAL	D-Maltose	+	+	+	+	+	+	100
dMAN	D-Mannitol	-	-	-	-	-	-	0
dMNE	D-Mannose	-	-	-	-	-	-	0
BXYL	Beta-Xylosidase	-	-	-	-	-	-	0
BAlap	Beta-Alanine arylamidase pNA	-	-	-	-	-	-	0
ProA	L-Proline Arylamidase	+	+	+	+	+	+	100
LIP	Lipase	-	+	-	-	-	-	16.67
PLE	Palatinose	-	-	-	-	-	-	0
TyrA	Tyrosine Arylamidase	+	+	+	+	+	+	100
URE	Urease	+	+	+	+	+	+	100
dSOR	D-Sorbitol	-	-	-	-	-	-	0
SAC	Saccharose/Sucrose	-	-	-	-	-	-	0
dTAG	D-Tagatose	-	-	-	-	-	-	0
dTRE	D-Trehalose	-	-	-	-	-	-	0
CIT	Citrate (Sodium)	-	-	-	-	-	-	0
MNT	Malonate	-	-	-	-	-	-	0
5KG	5-Keto-D-Gluconate	-	-	-	-	-	-	0
ILATk	L-Lactate alkalinisation	+	+	+	+	+	+	100
AGLU	Alpha Glucosidase	-	-	-	-	-	-	0
SUCT	Succinate Alkalinisation	+	+	+	+	-	-	66.67
NAGA	Beta-N-Acetyl Galactosaminidase	-	-	-	-	-	-	0
AGAL	Alpha Galactosidase	-	-	-	-	-	-	0
PHOS	Phosphatase	-	-	-	-	-	-	0
GlyA	Glycine Arylamidase	-	-	-	-	-	-	0
ODC	Ornithine Decarboxylase	-	-	-	-	-	-	0
LDC	Lysine Decarboxylase	-	-	-	-	-	-	0
lHISa	L-Histidine Assimilation	-	-	-	-	-	-	0
CMT	Coumarate	+	+	+	+	+	+	100
BGUR	Beta-Glucoronidase	-	-	-	-	-	-	0
O129R	O/129 Resistance	+	+	+	+	+	+	100
GGAA	Glu-Gly-Arg Arylamidase	-	+	+	(-)	-	+	66.67
IMLTa	L-Malate Assimilation	-	-	-	-	-	-	0
ELLM	Ellman	-	-	-	-	-	-	0
ILATa	L-Lactate Assimilation	-	-	-	-	-	-	0

Table of reference strains for constructing phylogenetic tree based on 16S rRNA sequences.

No.	Strain Name	Reference	No. accession
1.	*Escherichia coli* ATCC 35218	Unpublished	AM980865.1
2.	*Aeromonas caviae* ATCC 15467	Martinez-Murcia et al. (1992)	X60409 S42862
3.	*Aeromonas jandaei* ATCC 49568	Ruimy et al. (1994)	NR_119040
4.	*Aeromonas salmonicida* CECT 894T	Unpublished	AY987751.1
5.	*Aeromonas veronii* ATCC 35624	Unpublished	MK990549.1
6.	*Aeromonas hydrophila* ATCC 7966	Seshadri et al. (2006)	NR_075420.1
7.	*Aeromonas sobria* ATCC 43979	Ruimy et al. (1994)	NR_119044.1
8.	*Aeromonas schubertii* ATCC 43700	Ruimy et al. (1994)	NR_119043.1
9.	*Aeromonas schubertii* LMG 12668	Latif-Eugenín (2016)	LN849804.1
10.	*Aeromonas schubertii* LMG 12655	Latif-Eugenín (2016)	LN849805.1
11.	*Aeromonas schubertii* LF1708	Liu et al. (2018)	MG593845.1
12.	*Aeromonas schubertii* ZS20100725	Chen et al. (2012)	HQ407423.1
13.	*Aeromonas schubertii* ZS20100725-1	Chen et al. (2012)	HQ541164.1
14.	*Aeromonas schubertii* ZS20100725-2	Chen et al. (2012)	HQ541165.1
15.	*Aeromonas schubertii* ZL-1	Unpublished	MK410038.1
16.	*Aeromonas schubertii* ZL-13	Unpublished	MK410040.1
17.	*Aeromonas schubertii* WL-1	Unpublished	JQ303345.1
18.	*Aeromonas schubertii* WL-2	Unpublished	JQ319029.1
19.	*Aeromonas schubertii* HYL1	Liu and Li (2012)	GQ844300.1
20.	*Aeromonas schubertii* HYL2	Liu and Li (2012)	GQ844302.1
21.	*Aeromonas schubertii* HYK1	Liu and Li (2012)	GQ844301.1
22.	*Aeromonas schubertii* HYK2	Liu and Li (2012)	GQ844303.1
23.	*Aeromonas schubertii* HYB1	Liu and Li (2012)	GQ845450.1
24.	*Aeromonas schubertii* HYB2	Liu and Li (2012)	GQ845451.1
25.	*Aeromonas schubertii* HYS1	Liu and Li (2012)	GQ845452.1
26.	*Aeromonas schubertii* HYS2	Liu and Li (2012)	GQ845453.1

Table of reference strains for constructing phylogenetic tree based on *gyrB* sequences.

No.	Strain Name	Reference	No. accession
1.	*Escherichia coli* ATCC 35218	Delmas et al. (2006)	AY370845.1
2.	*Aeromonas caviae* ATCC 15467	unpublished	AB436662.1
3.	*Aeromonas jandaei* ATCC 49568	Aravena-Román et al. (2011)	FN706559.1
4.	*Aeromonas salmonicida* CECT 894T	Unpublished	AY987517.1
5.	*Aeromonas media* ATCC 39907	Aravena-Román et al. (2011)	FN706560.1

No.	Strain Name	Reference	No. accession
5.	*Aeromonas veronii* ATCC 35624	Pidiyar et al. (2003)	AF417626.1
6.	*Aeromonas hydrophila* ATCC 7966	Pidiyar et al. (2003)	AF417622.1
7.	*Aeromonas sobria* ATCC 9071	Aravena-Román et al. (2011)	FN796747.1
8.	*Aeromonas schubertii* ATCC 43701	Küpfer et al. (2006)	AJ868402.1
9.	*Aeromonas schubertii* LMG 12668	Latif-Eugenín (2016)	LN849798.1
10.	*Aeromonas schubertii* LMG 12655	Latif-Eugenín (2016)	LN849799.1
11.	*Aeromonas schubertii* MDC 575	Martinez-Murcia et al. (2011)	HQ442754.1
12.	*Aeromonas schubertii* MDC 574	Martinez-Murcia et al. (2011)	HQ442753.1
13.	*Aeromonas schubertii* BT3-772	Yano et al. (2015)	LC003078.1
14.	*Aeromonas schubertii* BT3-777	Yano et al. (2015)	LC003081.1
15.	*Aeromonas schubertii* WL-2	Unpublished	JQ319030.1
16.	*Aeromonas schubertii* WL-4	Unpublished	MK416159.1
17.	*Aeromonas schubertii* ZL-1	Unpublished	MK410039.1
19.	*Aeromonas schubertii* HYL1	Liu and Li (2012)	HQ731454.1
20.	*Aeromonas schubertii* HYL2	Liu and Li (2012)	HQ731455.1
21.	*Aeromonas schubertii* HYK1	Liu and Li (2012)	HQ731456.1
22.	*Aeromonas schubertii* HYK2	Liu and Li (2012)	HQ731457.1
23.	*Aeromonas schubertii* HYB1	Liu and Li (2012)	HQ731458.1
24.	*Aeromonas schubertii* HYB1	Liu and Li (2012)	HQ731459.1
25.	*Aeromonas schubertii* HYS1	Liu and Li (2012)	HQ731460.1
26.	*Aeromonas schubertii* HYS2	Liu and Li (2012)	HQ731461.1

Table of isolates used for MALDI ToF MS.

No.	isolates	Species	Host	Location	Year	Reference
1.	CUVETAHY01	*Aeromonas hydrophila*	Nile tilapia	Kanchanaburi, Thailand	2019	CE-FID CU
2.	CUVETAVE01	*Aeromonas veronii*	Seabass	Uttaradit, Thai-land	2018	CE-FID CU
3.	CUVETAJA01	*Aeromonas jandaei*	Nile tilapia	Kanchanaburi, Thailand	2019	CE-FID CU
4.	CUVETACA01	*Aeromonas caviae*	Nile tilapia	Kanchanaburi, Thailand	2019	CE-FID CU

No.	isolates	Species	Host	Location	Year	Reference
5.	CUVETASC03-08	*Aeromonas schubertii*	Snakehead fish	Nakhon Pathom, Thailand	2020	CE-FID CU

Table of semi-quantitative score of gross pathology lesions

No.	Score	Skin lesion	Internal hemorrhage	White nodules
1	0 – no changes	No visible change	No visible change	No visible change
2	1 – mild change	A localized lesion	Localized or 1 organ affected	1-3 visible white nodules
3	2 – moderate change	2-4 localized lesions	Multiple localized or 1-4 organs affected	4-10 visible white nodules
4	3 – severe change	Diffused or >4 localized lesions	Diffused or >4 organs affected	>10 visible white nodules

Table of semi-quantitative score of histopathology lesions

No.	Score	Melano-macrophage center (MMC)	Necrotic cells	granuloma
1	0 – No changes	No visible change	No visible change	No visible change
2	1 – mild change	1-3 visible MMC	Localized necrotic cells	1-3 visible granulomas
3	2 – moderate change	4-10 visible MMC	Multiple localized necrotic cells	4-10 visible granulomas
4	3 – severe change	>10 visible MMC	Diffused necrotic cells and tissue	>10 visible granulomas

Table of Recording Gross Pathology

			103						103'						104						104'						105						105'						106					
						WN						WN						WN						WN						WN						WN						WN		
No	date	time	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S
1	17/12/20	7:00																																										
		18:00																																										
2	18/12/20	7:00																																										
		18:00																																										
3	19/12/20	7:00																																										
		18:00																																										
4	20/12/20	7:00																																										
		18:00																																										
5	21/12/20	7:00																																										
		18:00																																										
6	22/12/20	7:00																																										
		18:00																																										
7	23/12/20	7:00																																										
		18:00																																										
8	24/12/20	7:00																																										
		18:00																																										
9	25/12/20	7:00																																										
		18:00																																										
10	26/12/20	7:00																																										
		18:00																																										
11	27/12/20	7:00																																										
		18:00																																										
12	28/12/20	7:00																																										
		18:00																																										
13	29/12/20	7:00																																										
		18:00																																										
14	30/12/20	7:00																																										
		18:00																																										
15	31/12/20	7:00																																										
		18:00																																										
16	1/1/2021	7:00																																										
		18:00																																										
17	2/1/2021	7:00																																										
		18:00																																										
18	3/1/2021	7:00																																										
		18:00																																										
19	4/1/2021	7:00																																										
		18:00																																										
20	5/1/2021	7:00																																										
		18:00																																										
21	6/1/2021	7:00																																										
		18:00																																										
	total																																											

			106'						107						107'						108						108'						control						control'					
						WN						WN						WN						WN						WN						WN						WN		
No	date	time	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S	D	SU	H	L	K	S
1	17/12/20	7:00																																										
		18:00																																										
2	18/12/20	7:00																																										
		18:00																																										
3	19/12/20	7:00																																										
		18:00																																										
4	20/12/20	7:00																																										
		18:00																																										
5	21/12/20	7:00																																										
		18:00																																										
6	22/12/20	7:00																																										
		18:00																																										
7	23/12/20	7:00																																										
		18:00																																										
8	24/12/20	7:00																																										
		18:00																																										
9	25/12/20	7:00																																										
		18:00																																										
10	26/12/20	7:00																																										
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11	27/12/20	7:00																																										
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12	28/12/20	7:00																																										
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13	29/12/20	7:00																																										
		18:00																																										
14	30/12/20	7:00																																										
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		18:00																																										
16	1/1/2021	7:00																																										
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17	2/1/2021	7:00																																										
		18:00																																										
18	3/1/2021	7:00																																										
		18:00																																										
19	4/1/2021	7:00																																										
		18:00																																										
20	5/1/2021	7:00																																										
		18:00																																										
21	6/1/2021	7:00																																										
		18:00																																										
	total																																											

Table of Recording Histopathology

No	organ	lesion	103												104											
			7 days				14 days				21 days				7 days				14 days				21 days			
			1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	Liver	Gran																								
2		MMC																								
3		NC																								
4	Kidney	Gran																								
5		MMC																								
6		NC																								
7	Spleen	Gran																								
8		MMC																								
9		NC																								
total																										

No	organ	lesion	105												106											
			7 days				14 days				21 days				7 days				14 days				21 days			
			1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	Liver	Gran																								
2		MMC																								
3		NC																								
4	Kidney	Gran																								
5		MMC																								
6		NC																								
7	Spleen	Gran																								
8		MMC																								
9		NC																								
total																										

No	organ	lesion	107												108											
			7 days				14 days				21 days				7 days				14 days				21 days			
			1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	Liver	Gran																								
2		MMC																								
3		NC																								
4	Kidney	Gran																								
5		MMC																								
6		NC																								
7	Spleen	Gran																								
8		MMC																								
9		NC																								
total																										

No	organ	lesion	control											
			7 days				14 days				21 days			
			1	2	3	4	1	2	3	4	1	2	3	4
1	Liver	Gran												
2		MMC												
3		NC												
4	Kidney	Gran												
5		MMC												
6		NC												
7	Spleen	Gran												
8		MMC												
9		NC												
total														

No	organ	lesion	103'												104'											
			7 days				14 days				21 days				7 days				14 days				21 days			
			1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	Liver	Gran																								
2		MMC																								
3		NC																								
4	Kidney	Gran																								
5		MMC																								
6		NC																								
7	Spleen	Gran																								
8		MMC																								
9		NC																								
total																										

No	organ	lesion	105'												106'											
			7 days				14 days				21 days				7 days				14 days				21 days			
			1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	Liver	Gran																								
2		MMC																								
3		NC																								
4	Kidney	Gran																								
5		MMC																								
6		NC																								
7	Spleen	Gran																								
8		MMC																								
9		NC																								
total																										

No	organ	lesion	107'												108'											
			7 days				14 days				21 days				7 days				14 days				21 days			
			1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	Liver	Gran																								
2		MMC																								
3		NC																								
4	Kidney	Gran																								
5		MMC																								
6		NC																								
7	Spleen	Gran																								
8		MMC																								
9		NC																								
total																										

No	organ	lesion	control'											
			7 days				14 days				21 days			
			1	2	3	4	1	2	3	4	1	2	3	4
1	Liver	Gran												
2		MMC												
3		NC												
4	Kidney	Gran												
5		MMC												
6		NC												
7	Spleen	Gran												
8		MMC												
9		NC												
total														

Appendix D Statistical analysis

Table of one-way ANOVA of mortality rate on each week with p-value 0.05

		Sum of Squares	df	Mean Square	F	Sig.
week1	Between Groups	261.061	6	43.510	4.710	0.001
	Within Groups	388.000	42	9.238		
	Total	649.061	48			
week2	Between Groups	1045.265	6	174.211	371.145	0.000
	Within Groups	19.714	42	0.469		
	Total	1064.980	48			
week3	Between Groups	1182.857	6	197.143	483.000	0.000
	Within Groups	17.143	42	0.408		
	Total	1200.000	48			

*week1: 7 dpi, week2: 14 dpi, week3: 21 dpi.

Table of Duncan post hoc test of mortality rates on each week.

Week 1 (7-dpi)

		Subset for alpha = 0.05	
dose	N	1	2
dose 104	7	0.000	
control	7	0.000	
dose 103	7	0.714	
dose 105	7	3.000	3.000
dose 106	7		5.000
dose 108	7		5.143
dose 107	7		5.429
Sig.		0.098	0.180

Week 2 (14-dpi)

		Subset for alpha = 0.05				
dose	N	1	2	3	4	5
control	7	0.000				
dose 103	7		1.000			
dose 104	7		1.286			
dose 105	7			8.571		
dose 108	7				9.857	
dose 107	7				10.429	10.429
dose 106	7					11.000
Sig.		1.000	0.440	1.000	0.126	0.126

Week 3 (21-dpi)

dose	N	Subset for alpha = 0.05				
		1	2	3	4	5
control	7	0.000				
dose 104	7		2.571			
dose 103	7		3.000			
dose 105	7			9.000		
dose 106	7				11.143	
dose 108	7					12.429
dose 107	7					12.857
Sig.		1.000	0.216	1.000	1.000	0.216

Table of Kruskal Wallis of Gross pathology lesions (Skin Ulcers, Hemorrhage and Granuloma) on each week (7, 14, and 21 dpi) and on each different dose of *A. schubertii* ($1.2x10^3$, 10^4, 10^5, 10^6, 10^7, 10^8, and control) with p–value < 0.05.

Week

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Chi-Square	16.934	30.781	22.907	11.800	9.276	10.410	17.335	19.494	20.800
df	2	2	2	2	2	2	2	2	2
Asymp. Sig.	0.000	0.000	0.000	0.003	0.010	0.005	0.000	0.000	0.000

Dose

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Chi-Square	31.503	25.499	32.590	33.464	43.338	39.315	43.797	38.672	36.873
df	6	6	6	6	6	6	6	6	6
Asymp. Sig.	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table of Mann Whitney U test of Gross pathology lesions on each week as mean comparison.

Week 1 (7-dpi) and Week 2 (14-dpi)

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	336.000	252.000	208.000	347.000	153.000
Wilcoxon W	742.000	658.000	614.000	753.000	559.000
Z	-1.134	-2.426	-3.178	-0.821	-4.128
Asymp. Sig. (2-tailed)	0.257	0.015	0.001	0.412	0.000

Week 1 (7-dpi) and Week 3 (21-dpi)

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	290.500	144.000	124.000	320.000	132.000
Wilcoxon W	696.500	550.000	530.000	726.000	538.000
Z	-1.968	-4.231	-4.552	-1.298	-4.463
Asymp. Sig. (2-tailed)	0.049	0.000	0.000	0.194	0.000

Week 2 (14-dpi) and Week 3 (21-dpi)

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	336.500	250.000	248.500	359.000	335.000
Wilcoxon W	742.500	656.000	654.500	765.000	741.000
Z	-1.034	-2.508	-2.519	-0.630	-0.996
Asymp. Sig. (2-tailed)	0.301	0.012	0.012	0.528	0.319

Table of Mann Whitney U test of Gross pathology lesions on each different dose of *A. schubertii* as mean comparison.

Control and dose 10^3

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	48.000	24.000	18.000	24.000	18.000
Wilcoxon W	126.000	102.000	96.000	102.000	96.000
Z	-2.145	-3.327	-3.625	-3.391	-3.616
Asymp. Sig. (2-tailed)	0.032	0.001	0.000	0.001	0.000
Exact Sig. [2*(1-tailed Sig.)]	.178b	.005b	.001b	.005b	.001b

Control and dose 10^4

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	48.000	18.000	12.000	30.000	18.000
Wilcoxon W	126.000	96.000	90.000	108.000	96.000
Z	-2.145	-3.625	-3.900	-3.077	-3.625
Asymp. Sig. (2-tailed)	0.032	0.000	0.000	0.002	0.000
Exact Sig. [2*(1-tailed Sig.)]	.178b	.001b	.000b	.014b	.001b

Control and dose 10^5

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	48.000	12.000	12.000	18.000	12.000
Wilcoxon W	126.000	90.000	90.000	96.000	90.000
Z	-2.138	-3.900	-3.890	-3.674	-3.890
Asymp. Sig. (2-tailed)	0.033	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.178b	.000b	.000b	.001b	.000b

Control and dose 10^6

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	42.000	12.000	12.000	18.000	12.000
Wilcoxon W	120.000	90.000	90.000	96.000	90.000
Z	-2.449	-3.909	-3.907	-3.674	-3.929
Asymp. Sig. (2-tailed)	0.014	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.089b	.000b	.000b	.001b	.000b

Control and dose 10^7

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	36.000	0.000	0.000	6.000	12.000
Wilcoxon W	114.000	78.000	78.000	84.000	90.000
Z	-2.753	-4.490	-4.480	-4.251	-3.916
Asymp. Sig. (2-tailed)	0.006	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.039b	.000b	.000b	.000b	.000b

Control and dose 10^8

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	24.000	0.000	0.000	0.000	6.000
Wilcoxon W	102.000	78.000	78.000	78.000	84.000
Z	-3.339	-4.494	-4.494	-4.529	-4.202
Asymp. Sig. (2-tailed)	0.001	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.005b	.000b	.000b	.000b	.000b

Dose 10^3 and dose 10^4

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	72.000	64.500	70.500	66.000	63.000
Wilcoxon W	150.000	142.500	148.500	144.000	141.000
Z	0.000	-0.466	-0.092	-0.413	-0.555
Asymp. Sig. (2-tailed)	1.000	0.641	0.927	0.680	0.579
Exact Sig. [2*(1-tailed Sig.)]	1.000b	.671b	.932b	.755b	.630b

Dose 10^3 and dose 10^5

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	70.000	62.500	63.000	62.000	55.000
Wilcoxon W	148.000	140.500	141.000	140.000	133.000
Z	-0.140	-0.579	-0.549	-0.700	-1.033
Asymp. Sig. (2-tailed)	0.889	0.562	0.583	0.484	0.301
Exact Sig. [2*(1-tailed Sig.)]	.932b	.590b	.630b	.590b	.347b

Dose 10^3 and dose 10^6

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	64.000	54.500	54.500	62.000	41.500
Wilcoxon W	142.000	132.500	132.500	140.000	119.500
Z	-0.544	-1.081	-1.081	-0.700	-1.877
Asymp. Sig. (2-tailed)	0.586	0.280	0.280	0.484	0.060
Exact Sig. [2*(1-tailed Sig.)]	.671b	.319b	.319b	.590b	.078b

Dose 10^3 and dose 10^7

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	58.000	34.500	45.000	42.000	33.000
Wilcoxon W	136.000	112.500	123.000	120.000	111.000
Z	-0.933	-2.304	-1.665	-2.079	-2.365
Asymp. Sig. (2-tailed)	0.351	0.021	0.096	0.038	0.018
Exact Sig. [2*(1-tailed Sig.)]	.443b	.028b	.128b	.089b	.024b

Dose 10^3 and dose 10^8

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	44.000	28.000	33.000	28.000	25.000
Wilcoxon W	122.000	106.000	111.000	106.000	103.000
Z	-1.803	-2.697	-2.427	-2.946	-2.816
Asymp. Sig. (2-tailed)	0.071	0.007	0.015	0.003	0.005
Exact Sig. [2*(1-tailed Sig.)]	.114b	.010b	.024b	.010b	.006b

Dose 10^4 and dose 10^5

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	70.000	70.500	65.000	56.500	63.000
Wilcoxon W	148.000	148.500	143.000	134.500	141.000
Z	-0.140	-0.092	-0.425	-1.055	-0.549
Asymp. Sig. (2-tailed)	0.889	0.927	0.671	0.292	0.583
Exact Sig. [2*(1-tailed Sig.)]	.932b	.932b	.713b	.378b	.630b

Dose 10^4 and dose 10^6

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	64.000	61.500	56.000	56.500	49.500
Wilcoxon W	142.000	139.500	134.000	134.500	127.500
Z	-0.544	-0.657	-0.975	-1.055	-1.427
Asymp. Sig. (2-tailed)	0.586	0.511	0.330	0.292	0.153
Exact Sig. [2*(1-tailed Sig.)]	.671b	.551b	.378b	.378b	.198b

Dose 10^4 and dose 10^7

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	58.000	40.500	46.500	37.500	39.000
Wilcoxon W	136.000	118.500	124.500	115.500	117.000
Z	-0.933	-1.965	-1.560	-2.316	-2.053
Asymp. Sig. (2-tailed)	0.351	0.049	0.119	0.021	0.040
Exact Sig. [2*(1-tailed Sig.)]	.443b	.068b	.143b	.045b	.060b

Dose 10^4 and dose 10^8

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	34.000	44.000	33.000	24.500	30.000
Wilcoxon W	112.000	122.000	111.000	102.500	108.000
Z	-2.372	-1.803	-2.427	-3.096	-2.550
Asymp. Sig. (2-tailed)	0.018	0.071	0.015	0.002	0.011
Exact Sig. [2*(1-tailed Sig.)]	.028b	.114b	.024b	.005b	.014b

Dose 10^5 and dose 10^6

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	66.500	63.000	64.000	72.000	60.000
Wilcoxon W	144.500	141.000	142.000	150.000	138.000
Z	-0.371	-0.552	-0.486	0.000	-0.736
Asymp. Sig. (2-tailed)	0.710	0.581	0.627	1.000	0.462
Exact Sig. [2*(1-tailed Sig.)]	.755b	.630b	.671b	1.000b	.514b

Dose 10^5 and dose 10^7

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	61.000	42.000	55.000	52.000	50.000
Wilcoxon W	139.000	120.000	133.000	130.000	128.000
Z	-0.726	-1.839	-1.033	-1.384	-1.336
Asymp. Sig. (2-tailed)	0.468	0.066	0.301	0.166	0.182
Exact Sig. [2*(1-tailed Sig.)]	.551b	.089b	.347b	.266b	.219b

Dose 10^5 and dose 10^8

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	48.000	35.000	44.000	37.500	41.000
Wilcoxon W	126.000	113.000	122.000	115.500	119.000
Z	-1.529	-2.258	-1.707	-2.316	-1.870
Asymp. Sig. (2-tailed)	0.126	0.024	0.088	0.021	0.062
Exact Sig. [2*(1-tailed Sig.)]	.178b	.033b	.114b	.045b	.078b

Dose 10^6 and dose 10^7

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	66.500	51.000	64.000	52.000	59.000
Wilcoxon W	144.500	129.000	142.000	130.000	137.000
Z	-0.357	-1.311	-0.492	-1.384	-0.828
Asymp. Sig. (2-tailed)	0.721	0.190	0.622	0.166	0.408
Exact Sig. [2*(1-tailed Sig.)]	.755b	.242b	.671b	.266b	.478b

Dose 10^6 and dose 10^8

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	53.000	43.000	52.000	37.500	48.500
Wilcoxon W	131.000	121.000	130.000	115.500	126.500
Z	-1.203	-1.808	-1.248	-2.316	-1.458
Asymp. Sig. (2-tailed)	0.229	0.071	0.212	0.021	0.145
Exact Sig. [2*(1-tailed Sig.)]	.291b	.101b	.266b	.045b	.178b

Dose 10^7 and dose 10^8

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Mann-Whitney U	58.000	63.000	59.000	56.500	61.000
Wilcoxon W	136.000	141.000	137.000	134.500	139.000
Z	-0.887	-0.566	-0.807	-1.055	-0.687
Asymp. Sig. (2-tailed)	0.375	0.571	0.420	0.292	0.492
Exact Sig. [2*(1-tailed Sig.)]	.443b	.630b	.478b	.378b	.551b

Table of Kruskal Wallis of Histopathology lesions (granuloma, melano-macrophage center (MMCs) and necrotic cells (NCs)) in each internal organs (liver, kidney and spleen) on each week (7, 14, and 21 dpi) and on each different dose of *A. schubertii* (1.2x103, 104, 105, 106, 107, 108, and control) with p–value < 0.05.

Week

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Chi-Square	4.128	20.047	24.542	1.872	25.120
df	2	2	2	2	2
Asymp. Sig.	0.127	0.000	0.000	0.392	0.000

Dose

	Liver white nodules	Kidney white nodules	Spleen white nodules	Skin ulceration	Hemorrhage
Chi-Square	4.128	20.047	24.542	1.872	25.120
df	2	2	2	2	2
Asymp. Sig.	0.127	0.000	0.000	0.392	0.000

Table of Mann-Whitney U test of Histopathology lesions of each internal organs in each week as mean comparison.

Week 1 (7-dpi) and Week 2 (14-dpi)

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	262.500	171.000	204.000	249.000	300.000	278.000	202.500	211.000	194.000
Wilcoxon W	668.500	577.000	610.000	655.000	706.000	684.000	608.500	617.000	600.000
Z	-2.343	-3.834	-3.247	-2.658	-1.611	-2.044	-3.284	-3.122	-3.428
Asymp. Sig. (2-tailed)	0.019	0.000	0.001	0.008	0.107	0.041	0.001	0.002	0.001

Week 1 (7-dpi) and Week 3 (21-dpi)

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	166.000	90.500	135.000	216.000	216.000	218.000	170.500	151.500	151.000
Wilcoxon W	572.000	496.500	541.000	622.000	622.000	624.000	576.500	557.500	557.000
Z	-3.951	-5.162	-4.399	-3.210	-3.149	-3.025	-3.791	-4.117	-4.125
Asymp. Sig. (2-tailed)	0.000	0.000	0.000	0.001	0.002	0.002	0.000	0.000	0.000

Week 2 (14-dpi) and Week 3 (21-dpi)

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	275.000	257.000	275.000	348.000	322.000	311.000	335.000	306.000	312.000
Wilcoxon W	681.000	663.000	681.000	754.000	728.000	717.000	741.000	712.000	718.000
Z	-2.070	-2.384	-2.101	-0.817	-1.266	-1.455	-0.996	-1.532	-1.439
Asymp. Sig. (2-tailed)	0.038	0.017	0.036	0.414	0.205	0.146	0.319	0.126	0.150

Table of Mann-Whitney U test of Histopathology lesions of each internal organs in each different dose of *A. schubertii* as mean comparison.

Control and dose 10^3

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	18.000	18.000	12.000	48.000	0.000	0.000	6.000	12.000	12.000
Wilcoxon W	96.000	96.000	90.000	126.000	78.000	78.000	84.000	90.000	90.000
Z	-3.715	-3.605	-3.890	-2.145	-4.522	-4.517	-4.226	-3.910	-3.900
Asymp. Sig. (2-tailed)	0.000	0.000	0.000	0.032	0.000	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.001b	.001b	.000b	.178b	.000b	.000b	.000b	.000b	.000b

Control and dose 10^4

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	18.000	12.000	6.000	48.000	0.000	0.000	6.000	12.000	12.000
Wilcoxon W	96.000	90.000	84.000	126.000	78.000	78.000	84.000	90.000	90.000
Z	-3.645	-3.900	-4.202	-2.145	-4.529	-4.517	-4.214	-3.932	-3.960
Asymp. Sig. (2-tailed)	0.000	0.000	0.000	0.032	0.000	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.001b	.000b	.000b	.178b	.000b	.000b	.000b	.000b	.000b

Control and dose 10^5

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	18.000	12.000	6.000	30.000	0.000	0.000	0.000	6.000	12.000
Wilcoxon W	96.000	90.000	84.000	108.000	78.000	78.000	78.000	84.000	90.000
Z	-3.625	-3.909	-4.187	-3.055	-4.529	-4.543	-4.494	-4.219	-3.929
Asymp. Sig. (2-tailed)	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.001b	.000b	.000b	.014b	.000b	.000b	.000b	.000b	.000b

Control and dose 10^6

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	12.000	12.000	6.000	18.000	0.000	0.000	0.000	0.000	6.000
Wilcoxon W	90.000	90.000	84.000	96.000	78.000	78.000	78.000	78.000	84.000
Z	-3.910	-3.894	-4.290	-3.715	-4.511	-4.543	-4.511	-4.494	-4.219
Asymp. Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.000b	.000b	.000b	.001b	.000b	.000b	.000b	.000b	.000b

Control and dose 10^7

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	12.000	12.000	6.000	12.000	0.000	0.000	0.000	0.000	0.000
Wilcoxon W	90.000	90.000	84.000	90.000	78.000	78.000	78.000	78.000	78.000
Z	-3.910	-3.894	-4.197	-3.932	-4.511	-4.511	-4.494	-4.480	-4.494
Asymp. Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.000b	.000b	.000b	.000b	.000b	.000b	.000b	.000b	.000b

Control and dose 10^8

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	0.000	6.000	6.000	6.000	0.000	0.000	0.000	0.000	0.000
Wilcoxon W	78.000	84.000	84.000	84.000	78.000	78.000	78.000	78.000	78.000
Z	-4.522	-4.184	-4.214	-4.226	-4.517	-4.488	-4.494	-4.488	-4.494
Asymp. Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Exact Sig. [2*(1-tailed Sig.)]	.000b	.000b	.000b	.000b	.000b	.000b	.000b	.000b	.000b

Dose 10^3 and dose 10^4

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	63.000	68.500	68.000	72.000	66.000	72.000	61.000	62.000	66.000
Wilcoxon W	141.000	146.500	146.000	150.000	144.000	150.000	139.000	140.000	144.000
Z	-0.626	-0.213	-0.245	0.000	-0.401	0.000	-0.709	-0.632	-0.382
Asymp. Sig. (2-tailed)	0.531	0.832	0.806	1.000	0.688	1.000	0.479	0.527	0.702
Exact Sig. [2*(1-tailed Sig.)]	.630b	.843b	.843b	1.000b	.755b	1.000b	.551b	.590b	.755b

Dose 10^3 and dose 10^5

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	58.500	60.500	65.000	52.000	66.000	66.500	53.000	51.000	63.000
Wilcoxon W	136.500	138.500	143.000	130.000	144.000	144.500	131.000	129.000	141.000
Z	-0.906	-0.702	-0.425	-1.316	-0.401	-0.371	-1.223	-1.325	-0.562
Asymp. Sig. (2-tailed)	0.365	0.483	0.671	0.188	0.688	0.710	0.221	0.185	0.574
Exact Sig. [2*(1-tailed Sig.)]	.443b	.514b	.713b	.266b	.755b	.755b	.291b	.242b	.630b

Dose 10^3 and dose 10^6

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	43.500	58.000	63.000	42.000	48.000	66.500	36.500	45.000	60.000
Wilcoxon W	121.500	136.000	141.000	120.000	126.000	144.500	114.500	123.000	138.000
Z	-1.857	-0.846	-0.572	-2.005	-1.560	-0.371	-2.247	-1.690	-0.752
Asymp. Sig. (2-tailed)	0.063	0.398	0.567	0.045	0.119	0.710	0.025	0.091	0.452
Exact Sig. [2*(1-tailed Sig.)]	.101b	.443b	.630b	.089b	.178b	.755b	.039b	.128b	.514b

Dose 10^3 and dose 10^7

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	43.500	58.000	61.000	30.000	48.000	53.000	27.000	35.000	39.000
Wilcoxon W	121.500	136.000	139.000	108.000	126.000	131.000	105.000	113.000	117.000
Z	-1.857	-0.846	-0.670	-2.660	-1.560	-1.240	-2.783	-2.268	-2.031
Asymp. Sig. (2-tailed)	0.063	0.398	0.503	0.008	0.119	0.215	0.005	0.023	0.042
Exact Sig. [2*(1-tailed Sig.)]	.101b	.443b	.551b	.014b	.178b	.291b	.008b	.033b	.060b

Dose 10^3 and dose 10^8

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	27.000	48.000	40.000	22.000	30.000	45.000	23.000	27.500	33.000
Wilcoxon W	105.000	126.000	118.000	100.000	108.000	123.000	101.000	105.500	111.000
Z	-3.021	-1.449	-1.950	-3.135	-2.689	-1.696	-2.989	-2.716	-2.363
Asymp. Sig. (2-tailed)	0.003	0.147	0.051	0.002	0.007	0.090	0.003	0.007	0.018
Exact Sig. [2*(1-tailed Sig.)]	.008b	.178b	.068b	.003b	.014b	.128b	.004b	.008b	.024b

Dose 10^4 and dose 10^5

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	67.500	63.000	68.500	52.000	72.000	66.500	63.000	61.000	68.000
Wilcoxon W	145.500	141.000	146.500	130.000	150.000	144.500	141.000	139.000	146.000
Z	-0.287	-0.552	-0.217	-1.316	0.000	-0.371	-0.570	-0.717	-0.267
Asymp. Sig. (2-tailed)	0.774	0.581	0.828	0.188	1.000	0.710	0.569	0.474	0.789
Exact Sig. [2*(1-tailed Sig.)]	.799b	.630b	.843b	.266b	1.000b	.755b	.630b	.551b	.843b

Dose 10^4 and dose 10^6

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	53.500	60.500	65.500	42.000	53.000	66.500	46.500	55.000	66.000
Wilcoxon W	131.500	138.500	143.500	120.000	131.000	144.500	124.500	133.000	144.000
Z	-1.164	-0.698	-0.436	-2.005	-1.254	-0.371	-1.641	-1.086	-0.401
Asymp. Sig. (2-tailed)	0.245	0.485	0.663	0.045	0.210	0.710	0.101	0.277	0.688
Exact Sig. [2*(1-tailed Sig.)]	.291b	.514b	.713b	.089b	.291b	.755b	.143b	.347b	.755b

Dose 10^4 and dose 10^7

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	53.500	60.500	63.500	30.000	53.000	53.000	35.000	43.000	42.000
Wilcoxon W	131.500	138.500	141.500	108.000	131.000	131.000	113.000	121.000	120.000
Z	-1.164	-0.698	-0.533	-2.660	-1.254	-1.240	-2.321	-1.810	-1.945
Asymp. Sig. (2-tailed)	0.245	0.485	0.594	0.008	0.210	0.215	0.020	0.070	0.052
Exact Sig. [2*(1-tailed Sig.)]	.291b	.514b	.630b	.014b	.291b	.291b	.033b	.101b	.089b

Dose 10^4 and dose 10^8

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	39.000	50.000	38.000	22.000	34.000	45.000	29.000	34.500	34.000
Wilcoxon W	117.000	128.000	116.000	100.000	112.000	123.000	107.000	112.500	112.000
Z	-2.126	-1.335	-2.122	-3.135	-2.480	-1.696	-2.633	-2.338	-2.372
Asymp. Sig. (2-tailed)	0.034	0.182	0.034	0.002	0.013	0.090	0.008	0.019	0.018
Exact Sig. [2*(1-tailed Sig.)]	.060b	.219b	.052b	.003b	.028b	.128b	.012b	.028b	.028b

Dose 10^5 and dose 10^6

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	58.500	68.500	70.000	64.500	53.000	72.000	57.000	66.000	70.000
Wilcoxon W	136.500	146.500	148.000	142.500	131.000	150.000	135.000	144.000	148.000
Z	-0.841	-0.215	-0.130	-0.510	-1.254	0.000	-0.941	-0.384	-0.130
Asymp. Sig. (2-tailed)	0.400	0.830	0.896	0.610	0.210	1.000	0.347	0.701	0.897
Exact Sig. [2*(1-tailed Sig.)]	.443b	.843b	.932b	.671b	.291b	1.000b	.410b	.755b	.932b

Dose 10^5 and dose 10^7

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	58.500	68.500	67.500	48.500	53.000	57.500	46.000	53.000	47.000
Wilcoxon W	136.500	146.500	145.500	126.500	131.000	135.500	124.000	131.000	125.000
Z	-0.841	-0.215	-0.278	-1.505	-1.254	-0.971	-1.603	-1.189	-1.586
Asymp. Sig. (2-tailed)	0.400	0.830	0.781	0.132	0.210	0.332	0.109	0.235	0.113
Exact Sig. [2*(1-tailed Sig.)]	.443b	.843b	.799b	.178b	.291b	.410b	.143b	.291b	.160b

Dose 10^5 and dose 10^8

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	45.000	58.000	43.500	39.500	34.000	48.500	40.000	44.000	39.000
Wilcoxon W	123.000	136.000	121.500	117.500	112.000	126.500	118.000	122.000	117.000
Z	-1.720	-0.860	-1.759	-2.075	-2.480	-1.500	-1.958	-1.752	-2.037
Asymp. Sig. (2-tailed)	0.085	0.390	0.079	0.038	0.013	0.134	0.050	0.080	0.042
Exact Sig. [2*(1-tailed Sig.)]	.128b	.443b	.101b	.060b	.028b	.178b	.068b	.114b	.060b

Dose 10^6 and dose 10^7

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	72.000	72.000	68.500	52.500	72.000	57.500	58.000	59.000	48.000
Wilcoxon W	150.000	150.000	146.500	130.500	150.000	135.500	136.000	137.000	126.000
Z	0.000	0.000	-0.234	-1.352	0.000	-0.971	-0.894	-0.807	-1.526
Asymp. Sig. (2-tailed)	1.000	1.000	0.815	0.177	1.000	0.332	0.371	0.420	0.127
Exact Sig. [2*(1-tailed Sig.)]	1.000b	1.000b	.843b	.266b	1.000b	.410b	.443b	.478b	.178b

Dose 10^6 and dose 10^8

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	60.000	62.000	38.500	42.000	53.000	48.500	49.000	50.000	40.000
Wilcoxon W	138.000	140.000	116.500	120.000	131.000	126.500	127.000	128.000	118.000
Z	-0.771	-0.608	-2.238	-2.077	-1.240	-1.500	-1.433	-1.366	-1.979
Asymp. Sig. (2-tailed)	0.441	0.543	0.025	0.038	0.215	0.134	0.152	0.172	0.048
Exact Sig. [2*(1-tailed Sig.)]	.514b	.590b	.052b	.089b	.291b	.178b	.198b	.219b	.068b

Dose 10^7 and dose 10^8

	Liver granuloma	Kidney granuloma	Spleen granuloma	Liver MMC	Kidney MMC	Spleen MMC	Liver necrotic cell	Kidney necrotic cell	Spleen necrotic cells
Mann-Whitney U	60.000	62.000	46.500	62.500	53.000	62.000	62.000	63.500	62.000
Wilcoxon W	138.000	140.000	124.500	140.500	131.000	140.000	140.000	141.500	140.000
Z	-0.771	-0.608	-1.598	-0.623	-1.240	-0.632	-0.625	-0.527	-0.625
Asymp. Sig. (2-tailed)	0.441	0.543	0.110	0.534	0.215	0.527	0.532	0.598	0.532
Exact Sig. [2*(1-tailed Sig.)]	.514b	.590b	.143b	.590b	.291b	.590b	.590b	.630b	.590b

Table of Mann-Whitney U test of white nodules of gross pathology lesions of each internal organs (liver, kidney, and spleen) and Granuloma of histopathology lesions of each internal organs with p-value < 0.05.

Liver White nodules and granuloma

	7-dpi	14-dpi	21-dpi
Mann-Whitney U	162.500	81.000	53.000
Wilcoxon W	462.500	381.000	353.000
Z	-2.884	-4.514	-5.072
Asymp. Sig. (2-tailed)	0.004	0.000	0.000

Kidney white nodules and granuloma

	7-dpi	14-dpi	21-dpi
Mann-Whitney U	244.000	159.000	110.000
Wilcoxon W	544.000	459.000	410.000
Z	-1.011	-2.918	-4.100
Asymp. Sig. (2-tailed)	0.312	0.004	0.000

Spleen white nodules and granuloma

	7-dpi	14-dpi	21-dpi
Mann-Whitney U	209.500	243.000	264.000
Wilcoxon W	509.500	543.000	564.000
Z	-1.772	-1.032	-0.606
Asymp. Sig. (2-tailed)	0.076	0.302	0.545

Table of Spearman Correlation between white nodules of each internal organs (liver, kidney, and spleen) and Granuloma of each internal organs with p-value < 0.05.

			Liver granuloma	Kidney granuloma	Spleen granuloma	Liver white nodules	Kidney white nodules	Spleen white
Spearman's rho	Liver granuloma	Correlation Coefficient	1.000	.718**	.704**	.510**	.721**	.724**
		Sig. (2-tailed)	.	0.000	0.000	0.000	0.000	0.000
		N	84.000	84.000	84.000	84.000	84.000	84.000
	Kidney granuloma	Correlation Coefficient	.718**	1.000	.796**	.507**	.826**	.844**
		Sig. (2-tailed)	0.000	.	0.000	0.000	0.000	0.000
		N	84.000	84.000	84.000	84.000	84.000	84.000
	Spleen granuloma	Correlation Coefficient	.704**	.796**	1.000	.407**	.777**	.770**
		Sig. (2-tailed)	0.000	0.000	.	0.000	0.000	0.000
		N	84.000	84.000	84.000	84.000	84.000	84.000
	Liver white nodules	Correlation Coefficient	.510**	.507**	.407**	1.000	.524**	.620**
		Sig. (2-tailed)	0.000	0.000	0.000	.	0.000	0.000
		N	84.000	84.000	84.000	84.000	84.000	84.000
	Kidney white nodules	Correlation Coefficient	.721**	.826**	.777**	.524**	1.000	.842**
		Sig. (2-tailed)	0.000	0.000	0.000	0.000	.	0.000
		N	84.000	84.000	84.000	84.000	84.000	84.000
	Spleen white nodules	Correlation Coefficient	.724**	.844**	.770**	.620**	.842**	1.000
		Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	.
		N	84.000	84.000	84.000	84.000	84.000	84.000

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