UNIVERSITI PUTRA MALAYSIA

BACTERIAL STOMATITIS IN WILD-CAUGHT RETICULATED PYTHONS (*Malayopython reticulatus*)

HO SHAO JIAN

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Bacterial Stomatitis in Wild-Caught Reticulated Pythons (*Malayopython reticulatus*)" by Ho Shao Jian and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Final Year Project.

DR. AZLAN CHE' AMAT

DVM, MVSc (UPM), PhD (UCLM Spain),

Senior Lecturer,

Department of Veterinary Clinical Studies,

Faculty of Veterinary Medicine,

Universiti Putra Malaysia

(Main Supervisor)

DR. SHARINA OMAR

DVM (UPM), MSc (Massey University), PhD (University of Leicester),

Senior Lecturer,

Department of Veterinary Pathology & Microbiology,

Faculty of Veterinary Medicine,

Universiti Putra Malaysia

(Co-Supervisor)

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LIST OF ABBREVIATIONS

AST	antimicrobial susceptibility testing
CLSI	Clinical and Laboratory Standards Institute
LDC	lysine decarboxylase
MDR	multi-drug resistant
ODC	ornithine decarboxylase
OF	oxidation-fermentation
ONPG	O-Nitrophenyl-β-D-galactopyranoside
SIM	sulfide-indole-motility
sp.	species
spp.	species
TSI	triple sugar iron

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Akhir Tahun.

BAKTERIA PENYEBAB STOMATITIS DALAM ULAR SAWA BATIK (*Malayopython reticulatus*) LIAR YANG DITANGKAP

Oleh

Ho Shao Jian

2020

Penyelia Utama: Dr. Azlan Che' Amat

Penyelia Bersama: Dr. Sharina Omar

Stomatitis bakteria adalah penyakit klinikal saluran gastrousus atas yang paling biasa dalam reptilia. Kajian ini bertujuan untuk menjalankan isolasi dan pengenalpastian bakteria aerob dalam kaviti oral ular sawa batik yang mempunyai atau tidak mempunyai stomatitis serta menguji kerentanan antimikrobial mereka. Sejumlah N=40 bangkai ular sawa batik telah dinilai untuk kehadiran atau ketiadaan stomatitis. Calitan oral telah diambil dan dikultur atas agar darah dan MacConkey. Morfologi koloni dan selular telah dinilai, diikuti oleh ujian biokimia seperti ujian katalase dan oksidase untuk peneganalpastian spesies. Ujian kerentanan antimikrobial telah dijalankan atas bakteria yang dikenalpasti dengan menggunakan kaedah penyebaran cakera Kirby-Bauer terhadap gentamicin, amoxicillin, cephalexin, azithromycin, tetracycline dan ciprofloxacin. Kajian ini mendapati kelaziman stomatitis adalah 77.5% (31/40). Kelaziman stomatitis dalam ular sawa jantan dan betina mempunyai perbezaan yang signifikan ($p < 0.05$). Sejumlah 153 organisma telah dikenalpasti, dengan bakteria gram-negatif menjadi dominan. Spesies bakteria yang paling kerap diisolasi adalah *Aeromonas* sp. (14.4%; 22/153), diikuti oleh *Klebsiella pneumoniae* (11.8%, 18/153) dan *Alcaligenes faecalis* (8.5%; 13/153). *Staphylococcus* koagulase-negatif (4.6%; 7/153) dan *Corynebacterium* sp. (0.7%; 1/153) merupakan satu-satunya aerob gram-positif yang diisolasi. Kebanyakan organisma mempunyai kerentanan antibiotik yang sama terhadap gentamicin dan ciprofloxacin (95.8%) serta perintang antibiotik yang tinggi terhadap amoxicillin (83.3%) dan cephalexin (75.0%). Kesimpulannya, backteria penyebab stomatitis dalam ular sawa batik liar yang ditangkap adalah sangat lazim dengan kebiasaannya isolasi bakteria campuran, serta gentamicin dan ciprofloxacin harus dipertimbangkan sebagai antibiotik pilihan bagi rawatan penyakit ini.

Kata kunci: liar, ular sawa batik, bakteria, stomatitis, ujian kerentanan antimikrobial

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

BACTERIAL STOMATITIS IN WILD-CAUGHT RETICULATED PYTHONS (*Malayopython reticulatus*)

By

Ho Shao Jian

2020

Main Supervisor: Dr. Azlan Che' Amat

Co-Supervisor: Dr. Sharina Omar

Bacterial stomatitis is considered as the most common clinical form of upper alimentary tract disease in reptiles. This study aimed to isolate and identify the common aerobes in the oral cavities of wild-caught reticulated pythons presented with or without stomatitis and to profile their antimicrobial susceptibility. A total of N=40 carcasses of the wild-caught reticulated pythons were assessed for the presence or absence of stomatitis. Oral swabs were obtained and cultured onto blood and MacConkey agar. The colony and cellular morphologies of the isolates were evaluated, followed by biochemical tests such as catalase and oxidase tests for species identification. Antimicrobial susceptibility testing was performed on the identified isolates using Kirby-Bauer disk diffusion method against gentamicin, amoxicillin, cephalexin, azithromycin, tetracycline, and ciprofloxacin. This study discovered that the prevalence of stomatitis was 77.5% (31/40). The occurrence of stomatitis was significantly ($p < 0.05$) different between the male and female pythons. A total of 153 isolates were identified, with gram-negative bacteria being predominant. The most isolated bacterial species was *Aeromonas* sp. (14.4%; 22/153), followed by *Klebsiella pneumoniae* (11.8%; 18/153), and *Alcaligenes faecalis* (8.5%; 13/153). Coagulase-negative *Staphylococcus* (4.6%; 7/153) and *Corynebacterium* sp. (0.7%; 1/153) were the only gram-positive aerobes isolated. Most of the isolates were equally susceptible to gentamicin and ciprofloxacin (95.8%) and highly resistant to amoxicillin (83.3%) and cephalexin (75.0%). In conclusion, bacterial stomatitis in wild-caught reticulated pythons is highly prevalent with commonly a mixed bacterial isolation, and gentamicin as well as ciprofloxacin should be considered as the primary line of antibiotics in the treatment of this disease.

Keywords: wild, reticulated python, bacteria, stomatitis, antimicrobial susceptibility testing

CHAPTER 1

INTRODUCTION

1.1 Background of study

The reticulated python (*Malayopython reticulatus*) is the world's longest snake in the family of Pythonidae. It has been described as the most important species among other pythons from the economic aspects (Groombridge & Luxmoore, 1990). Pythons have been exploited for the purposes of products to be sold in fashion, food, and traditional medicine markets (Kasterine *et al.*, 2012; Klemens & Thorbjarnarson, 1995). In Southeast Asia, approximately 340,000 skins of reticulated python are exported annually, making it to be the most heavily traded species in the trade of python skins. Malaysia is considered to be one of the main sources of pythons for the skin trade, alongside with Indonesia, most of which are wild-caught (Kasterine *et al.*, 2012).

Bacterial and *Mycoplasma* spp. infections are frequently reported among the reptiles. Bacterial infections caused by gram-negative bacteria are more common in reptilian diseases. However, gram-negative bacteria are usually the normal flora found in reptiles, so their existence is not necessary an indication to diseases (O'Rourke & Schumacher, 2015). Besides, gram-positive bacteria, anaerobes and *Mycoplasma* spp. play a crucial role in reptilian diseases, too (Rosenthal & Mader, 1996). Several published literatures stated that examples of gram-positive bacteria reside predominantly in the oral cavities of healthy reptiles are *Corynebacterium* and *Staphylococcus*, while examples of gram-negative bacteria which are predominant in the oral cavities of reptiles with stomatitis are *Pseudomonas* sp. and *Aeromonas* sp. (Johnson & Benson, 1996; McNamara *et al.*, 1994; Stewart, 1990). A more recent study discovered that mostly gram-negative bacteria were isolated in pythons as the normal flora, with *Aeromonas* spp. being the most isolated bacteria, followed by *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* (Abba *et al.*, 2017). *Bacteroides* spp. are the most common anaerobic isolates in reptiles, while *Clostridium* spp. have been described to have correlations with gastrointestinal disease and endotoxemia (Schmidt *et al.*, 2013).

Stomatitis is considered to be an important disease in captive snakes classified in the families of Boidae and Pythonidae. Infectious stomatitis is a common disease in snakes kept in captivity, which is also known as "mouth rot" (Diaz-Figueroa & Mitchell, 2006). A study on the aerobes that lead to stomatitis in snakes had been done, with the findings of gram-negative bacteria being the most isolated microorganisms, such as *E. coli*, *Citrobacter* spp., *Proteus* spp. and *Salmonella* spp., while *Staphylococcus* spp. being the only isolated gram-positive bacterium (Pereira *et al.*, 2017). Risk factors of stomatitis are infestation with mites, malnutrition, oral trauma, poor oral hygiene, neoplasia, inappropriate husbandry, and stress (Mehler & Bennett, 2006; Kaplan & Jereb, 1995). Untreated stomatitis may have high chances to progress to other diseases such as osteomyelitis and pneumonia (Jacobson, 2007; Mehler & Bennett, 2006). Osteomyelitis is thought to be resulted from chronic proliferative lesions which extend into the maxilla or mandible (Mehler & Bennett, 2006), while pneumonia is described to be caused by the presence of cellular debris in the respiratory tract through inhalation of the debris (Jacobson, 2007). Antimicrobial resistance among the bacterial isolates of captive reptiles had also been reported. For example, 9% of the *Salmonella* strains isolated from captive reptiles were resistant to several antimicrobial agents, such as ampicillin, amoxicillin/clavulanic acid, and streptomycin (Romero *et al.*, 2016).

Studies on the microbial flora of snakes in Malaysia are scanty. Moreover, there is lack of information on the common aerobic bacteria which cause stomatitis in the wild reticulated python with respect to the risk factors of stomatitis in Malaysia. Apart from that, there is also paucity of reports on the correlation of the occurrence of stomatitis in the wild reticulated python and the oral bacteria in Malaysia.

1.2 Objectives

The objectives of this project are:

i) To isolate and identify the common aerobic bacteria in the oral cavities of wild-caught reticulated pythons with or without stomatitis.

ii) To perform antimicrobial susceptibility testing (AST) on the isolated and identified bacteria which cause stomatitis in the wild-caught reticulated pythons.

1.3 Justification

There is insufficient information on the common aerobic bacteria which cause stomatitis in the wild reticulated python with respect to the risk factors of stomatitis in Malaysia. Furthermore, there is also paucity of reports on the correlation of the occurrence of stomatitis in the wild reticulated python and the oral bacteria in Malaysia. The AST is also important to determine the occurrence of antimicrobial resistance in the bacteria isolated from the lesions of stomatitis in the wild reticulated python in Malaysia.

1.4 Hypothesis

Null hypothesis: There is no association between the oral bacteria and the occurrence of stomatitis in the wild-caught reticulated pythons.

Alternative hypothesis: There is an association between the oral bacteria and the occurrence of stomatitis in the wild-caught reticulated pythons.

CHAPTER 2

LITERATURE REVIEW

2.1 Reticulated python (*Malayopython reticulatus*)

The reticulated python (*Malayopython reticulatus*), formerly known as *Python reticulatus*, is a non-venomous constrictor in the family of Pythonidae. It is the world's longest snake native to South Asia and Southeast Asia (Tan *et al.*, 2019). It is listed as "Least Concern" on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species as it is widely distributed in these two areas. Additionally, it can adapt to and is abundant in certain areas where it is exploited heavily. It has a wide range of habitats, mainly in terrestrial and freshwater systems. It can be found in rainforest, shrubland, and even near or in lakes or streams. The species had been reported to inhabit residential areas, where it is in close proximity with humans (Stuart *et al.*, 2018).

Malayopython reticulatus is the species with the most economic importance among other pythons (Groombridge & Luxmoore, 1990). Heavy exploitations of pythons have been carried out as they have been demanded for the markets of fashion, food, traditional medicine, and pet (Stuart *et al.*, 2018; Kasterine *et al.*, 2012; Klemens & Thorbjarnarson, 1995). In Southeast Asia, the reticulated python is the most heavily traded species for its skin, with approximately 340,000 skins being exported annually. Malaysia and Indonesia are considered as the main sources of pythons for the skin trade, most of which are caught from the wild (Kasterine *et al.*, 2012). In Peninsular Malaysia, the annual export quota of skins is 155,000 according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2018. Trade of the python skins are usually via neighbouring countries (Natusch *et al.*, 2016).

2.2 Oral cavity of the reticulated python

The oral cavity of the reticulated python consists of a few sets of salivary glands which include palatine, lingual, sublingual, and labial glands. The glands function to moisten the oral cavity and lubricate the preys (Frunk & Bogan, 2019). The tongue is a long, slender, mobile, and forked structure which lies in a sheath beneath the epiglottis and glottis. Protrusion of the tongue can be done without the snake opening its oral cavity through the lingual notch or fossa. It is a sensory organ which functions in olfaction, taste, and touch (O'Malley, 2005). The tongue tips could reach a groove in the upper jaw which the epiglottis fits into when the oral cavity is closed. The openings of the vomeronasal or Jacobson's organs are cranial to this groove. Snakes may not feed when they lose their tongues due to traumatic injury or infection (Frunk & Bogan, 2019).

There are six rows of teeth in the oral cavity, with one row on each of the lower jaws and two rows on each maxillary and palatine or pterygoid bones of the upper jaw (Edmund, 1970). The teeth function entirely in food prehension as snakes swallow their prey as a whole without having to masticate the prey into smaller pieces. The teeth are elongated, thin, pointed, and slightly curved caudally to prevent the prey from escaping (Frunk & Bogan, 2019). All snakes have pleurodont teeth that are attached to the medial jawbone. The teeth are continuously being replaced by new reserved teeth lying in the gums throughout life. Each tooth is shed every few months and swallowed together with the prey during feeding (Frunk & Bogan, 2019; O'Malley, 2005).

2.3 Stomatitis in snakes

Stomatitis which is also known as "mouth rot" is a condition characterized by inflammation and infection of the tissues of the oral cavity. It is a common and important disease in reptiles (Mehler & Bennett, 2006). Snakes affected by stomatitis exhibit clinical signs of anorexia, swelling around or in the oral cavity, erosion and abscessation of the oral mucosa, excessive salivation, disrupted lip margins, inability to properly close the mouth, hyperaemic oral mucosa, petechial haemorrhage, ulcerative lesion, mucosal plaques, decreased or no tongue flicking, as well as dysfunction of the larynx and associated structures (De Voe, 2019; Jho *et al.*, 2011b). In advanced cases which the treatment of stomatitis is absent, delayed, or ineffective, organs other than the oral cavity will demonstrate pathology, too. The possible consequences are swelling of the head may occur, the jaw, cranium or both may be affected, loosened teeth, enteritis resulted from ingestion of necrotic material from the oral plaques, osteomyelitis due to chronic proliferative lesions extending into the maxilla and mandible, pneumonia as a result of aspiration of the cellular debris or bacteria, and swelling of the eyes caused by ascending infection through the nasolacrimal duct. The most severe sequelae of stomatitis are septicaemic condition and death (Jho *et al.*, 2011b; Jacobson, 2007; Mehler & Bennett, 2006).

Mehler and Bennett (2006), together with De Voe (2019) stated that stomatitis is commonly regarded as a secondary condition following exposure to various predisposing factors rather than a primary condition itself. In addition, snakes that live in poor husbandry or environmental conditions, have traumatic injury, mite infestation or neoplasia, or are immunocompromised or malnourished, are more at risk of developing stomatitis. The authors also claimed that the nature of rubbing or crushing into barriers and capturing preys predisposed the snakes to traumatic injury in the oral cavity, leading to stomatitis resulting from desiccated and devitalized mucous membrane. They added that one of the examples of malnourishment is hypovitaminosis A, which it could lead to squamous metaplasia of the oral mucosa and glandular epithelium. Apart from mite infestation, *Amblyomma* ticks which can be commonly found in snakes, serve as the vectors for *Aeromonas hydrophila* that is able to lead to bacterial stomatitis in snakes (Catherine *et al.*, 2017).

De Voe (2019) explained that the treatment of stomatitis aims at providing appropriate husbandry conditions and long-term antimicrobial treatment. Appropriate husbandry conditions are crucial in reducing stress in reptiles which serve as a predisposition to the development of stomatitis. Supportive treatments might be required if the animal is anorexic or hypophagic. Besides, De Voe also stated that surgical debridement of the infected tissue and bone, wound cleaning as well as administration of non-steroidal anti-inflammatory drugs are also important in the treatment. He further added that the selection of antibiotics should be based on culture and sensitivity testing. Fluoroquinolones and aminoglycosides are the reasonable empirical antibiotics to be administered as most of the causative agents are gram-negative bacteria.

2.4 Causative agents of bacterial stomatitis

Bacterial stomatitis is a component of infectious stomatitis. Infectious stomatitis is considered to be the most common clinical form of upper alimentary tract disease in reptiles (Mehler & Bennett, 2006). The oral cavity of snakes is abundant with bacterial flora that can lead to opportunistic infection when the host is immunosuppressed, including bacterial stomatitis. The stomatitis-causing bacteria are also ubiquitous in the environment, which can easily be picked up by the snakes (Abba *et al.*, 2017).

Majority of the bacterial flora in the oral cavity of snakes are gram-negative aerobic bacteria (Lukač *et al.*, 2017; Dipineto, 2014; Lam *et al.*, 2010; Shek *et al.*, 2009; Blaylock, 2001). The examples of routinely isolated gram-negative aerobes are *Aeromonas hydrophila, Pseudomonas* sp., *E. coli*, *Stenotrophomonas maltophilia*, *Salmonella* sp., *Klebsiella* sp., *Serratia* sp., and *Providencia* sp. (Jho *et al.*, 2011a; Mehler & Bennett, 2006). Mehler & Bennett (2006) state that in the oral cavity of healthy snakes as well as snakes presented with stomatitis, the common bacterial isolates are *A. hydrophila*, *E. coli*, *Pseudomonas* sp., *Salmonella* sp., *Providencia* sp., *Klebsiella* sp., and *Proteus* sp. Moreover, in the captive and natural environments of snakes, *A. hydrophila*, *E. coli*, *Pseudomonas* sp., and *Proteus* sp. are routinely isolated from the water source (Hilf *et al.*, 1990). However, the differentiation of contamination of the environment by the snakes which subsequently lead to infection when they are immunosuppressed from contamination of the environment when the snakes are primarily infected is difficult (Mehler & Bennett, 2006). Gram-negative aerobic bacteria are predominant in the oral cavity of snakes presented with stomatitis as reported by Pereira *et al.* (2017). Among the stomatitis-causing gram-negative aerobic bacteria in snakes, *Pseudomonas* sp., *Aeromonas* sp., *Serratia* sp., *Salmonella* sp., *Proteus* sp., and *E. coli* are described as the common isolates (Jho *et al*., 2011b).

According to Wellehan and Drivers (2019), *A. hydrophila* and *Pseudomonas* sp. are gram-negative rod-shaped bacteria mainly found in water; *Pseudomonas* sp. can also be found in soil. They are opportunistic organisms and tend to have extensive resistance to antimicrobial agents. Therefore, antimicrobial sensitivity testing (AST) is important to identify which antibiotics they are sensitive to. Aminoglycosides can be used as the first-line antibiotics while the results of AST are pending. For aminoglycoside-resistant *Pseudomonas* sp., third generation cephalosporins or fluoroquinolones may be opted. Besides, Wellehan and Drivers described that *S. maltophilia* is an opportunist that can be discovered in water and soil. It has a high antimicrobial resistance rate, with trimethoprim-sulfamethoxazole being the first-line antibiotic while waiting for the AST results.

Salmonella sp., *Klebsiella* sp., *E. coli*, *Serratia* sp., *Providencia* sp., and *Proteus* sp. are in the family of Enterobacteriaceae, and all of them are gram-negative rod-shaped bacteria. In reptiles, the most common subspecies of *Salmonella* sp. is *Salmonella enterica* subspecies *enterica*, followed by subspecies *diarizone* and subspecies *arizonae*. It can cause salmonellosis in animals as well as humans who keep reptiles as their pets (Romero *et al*., 2016). *Salmonella* sp. also shows resistance to various antimicrobial agents (Chen *et al*., 2010). One of the species of *Klebsiella* sp. that is of public health concern is *K. pneumoniae*. It is capable of causing a variety of diseases in humans, including pneumonia and septicaemia; additionally, it is a gram-negative opportunistic bacterium, and has been reported to have an extensive resistance profile (Wang & He, 2018). Snakes have a higher frequency of isolation *E. coli* than other reptiles as all snakes are carnivorous animals; the type of diet and contact with other animals greatly influence the frequency of isolation of the organism (Ramos *et al*., 2019). The organism had also been reported to be resistant to antibiotics such as amoxicillin/clavulanic acid, tetracycline, etc. (Casey *et al*., 2015).

Serratia sp. is an opportunist that can cause nosocomial infection in human patients as well as diseases in animals. *Serratia* sp. have intrinsic resistance to penicillin G, macrolides, clindamycin, linezolid, glycopeptides, quinupristin-dalfopristin, and rifampin. Additionally, most of the members of *Serratia* sp. show resistance to ampicillin, amoxicillin, amoxicillin/clavulanic acid, ampicillin-sulbactam, narrow-spectrum cephalosporins, cephamycins, cefuroxime, nitrofurantoin, and colistin (Khanna *et al*., 2013; Mahlen, 2011). *Providencia* sp. is an opportunistic bacterium. The important species of the organism in reptiles is *Providencia rettgeri*. Crocodilians are the most heavily affected animals by this organism due to large epizootics and high mortality rates. Common pathological findings caused by *P. rettgeri* are sepsis, pneumonia, meningoencephalitis, splenitis, and hepatitis. *P. rettgeri* usually exhibits high antimicrobial resistance (Wellehan & Divers, 2019). *Proteus vulgaris*, *Proteus mirabilis*, and *Proteus penneri* had been isolated from the oral cavity of snakes as normal flora. *Proteus* sp. is an opportunist that can cause both humans and animals to be infected. Antimicrobial resistant *Proteus* sp. had also been documented (Drzewiecka, 2016).

Gram-positive bacteria are predominant in the oral cavity of healthy reptiles, with *Corynebacterium* and *Staphylococcus* being the common organisms. *Corynebacterium* is described as club-shaped rods that can be found in soil and skin flora. Isolation of this organism usually is not clinically relevant as this might be due to contamination, although *Corynebacterium* infections had been reported in reptiles. *Staphylococcus* is a genus of bacteria characterized by clustered gram-positive cocci. This organism is considered rare in causing diseases in reptiles (Wellehan & Divers, 2019). However, a case of methicillin-resistant *Staphylococcus aureus* (MRSA) subspectacular abscess had been reported in a Burmese python (*Python molurus bivittatus*), which is of high public health concern (Lee & Kim, 2011).

Anaerobic bacteria are also considered as the normal flora of reptiles and can be found in the oral cavity, skin, conjunctiva, nasal cavity, gastrointestinal tract, and faeces. The common anaerobes in reptiles are species of *Bacteroides*, *Clostridium*, *Fusobacterium*, and *Peptostreptococcus*. They can lead to a variety of diseases, ranging from oral abscesses and tissue necrosis to severe systemic disease. *Bacteroides* sp. was found to be the most common organism from bacterial cultures of abscesses in reptiles. *Bacteroides* spp. frequently show resistance to penicillin and tetracyclines (Mehler & Bennett, 2006). *Clostridium* is an anaerobic spore-forming gram-positive rod. The important species of disease-causing *Clostridium* in reptiles are *C. perfringens*, *C. glycolicum*, and *C. novyi*. Most of the species are susceptible to metronidazole; therefore, metronidazole is considered as the first-line antibiotic to treat the infections caused by this organism. *Fusobacterium necrophorum* is a non-spore forming anaerobe which is usually sensitive to metronidazole (Wellehan & Divers, 2019). *Peptostreptococcus* is a genus of anaerobic, gram-positive, non-spore forming bacteria, which has been shown to have increasing antimicrobial resistance (Könönen *et al.*,2007).

The common species of *Mycobacterium* in reptiles are *M. marinum*, *M. chelonei*, and *M. thamnopeos*. In reptiles, *Mycobacterium* sp. can result in diseases in the oral cavity which are characterized by chronic granulomatous or non-granulomatous lesions. The lesions may become haemorrhagic and can subsequently progress to osteomyelitis. Enrofloxacin, rifampin, and clarithromycin are administered as a combination to treat infections caused by *Mycobacterium* sp. However, treatment of mycobacteriosis in reptiles has been unsuccessful. Euthanasia should be considered due to the lack of success of treatment, chronic debilitating nature of the disease, and the zoonotic potential of mycobacteriosis (Mehler & Bennett, 2006).

2.5 Antibiotics used in reptiles

Aminoglycosides are the gold standard for sepsis caused by gram-negative bacteria. They are typically used to treat gram-negative bacterial infections. The combination of aminoglycosides and penicillins can also treat gram-positive bacterial infections. However, contraindications of administering aminoglycosides such as gentamicin include compromised renal function and dehydration as nephrotoxicity can be resulted. Amikacin is less nephrotoxic than gentamicin (Mitchell, 2006).

Cephalosporins can be divided into four generations. The first-generation cephalosporin has the narrowest spectrum of activity which covers gram-positive aerobic and anaerobic bacteria while the third-generation cephalosporin has the broadest spectrum against gram-negative bacteria. In patients presented with compromised renal function, cephalothin, a first-generation cephalosporin has been linked with the occurrence of renal disease as this drug undergoes hepatic metabolism and renal excretion (Mitchell, 2006; Plumb, 2002). Therefore, it is more suitable to use cefoperazome which is a third-generation cephalosporin in patients with pre-existing renal disease as this antibiotic is excreted by the liver (Mitchell, 2006).

Chloramphenicol is effective against gram-positive and gram-negative aerobes and anaerobes. It is primarily excreted via the hepatic pathway (Jacobson, 2000). A combination of chloramphenicol and ampicillin has been shown to be effective in the treatment of salmonellosis in lizards and chelonians (Koopman, 1976). Fluoroquinolones have been widely in human and veterinary medicine, with spectrum of activity against gram-positive and gram-negative bacteria. However, it is not very effective against anaerobes. Its primary excretion pathway is through kidney. Interestingly, enrofloxacin can be biotransformed into ciprofloxacin. Adverse effects including arthropathies and nervous problem are commonly reported in species other than reptiles (Mitchell, 2006).

Macrolides typically have lower effectiveness against anaerobes; however, majority of the anaerobic isolates from the clinical samples in snakes were susceptible to clindamycin (Stewart, 1990). Partial metabolization of this class of antibiotic is carried out by the liver, with unchanged excretion in bile (Mitchell, 2006). Metronidazole is a routine antibiotic used in treatments of protozoal and anaerobic bacterial infections. It is metabolized via hepatic pathway and excreted in the urine and faeces. The drug can adversely affect the nervous system, liver, and gastrointestinal tract if the dose causes toxicity. Anaerobic bacteria can gain antimicrobial resistance due to the increased use of metronidazole as prophylaxis or antiprotozoal drug (Mitchell, 2006).

Semisynthetic penicillins are effective against gram-negative bacteria. One of the examples of this class of antibiotic is carbenicillin. It had shown success in treatments of infections caused by *Pseudomonas* sp., but there is an increase in the resistance of the organism to this antibiotic (Lawrence, 1983). Tetracyclines have a broad spectrum of activity against aerobes. It has also been demonstrated that anaerobes from reptiles are sensitive to this class of antibiotic. This drug is excreted via the kidney or gastrointestinal tracts without being metabolized. *Salmonella* sp. and *P. aeruginosa* are intrinsically resistant to tetracyclines.

Increased resistance among the bacteria to this antibiotic had also been reported (Mitchell, 2006). Trimethoprim-sulfamethoxazole is a type of potentiated sulfonamides. It has a wide spectrum of activity against bacteria and protozoa in reptiles. Metabolization and excretion of this drug is via hepatic pathway and kidney, respectively. This drug can potentially lead to side effects such as hypersensitivity and crystalluria. The use of this drug is contraindicated when the patient is dehydrated (Mitchell, 2006).

2.6 Antimicrobial resistance threats

A considerable number of bacteria has been reported to develop antimicrobial resistance. The Centers for Disease Control and Prevention (CDC) in 2013 had categorized the antimicrobial resistant bacteria into three categories, which are urgent threats, serious threats, and concerning threats. Bacteria grouped in the category of urgent threats include *Clostridium difficile*, carbapenem-resistant Enterobacteriaceae (CRE), and drug-resistant *Neisseria gonorrhoeae*; in the category of serious threats include multidrug-resistant *Acinetobacter*, drug-resistant *Campylobacter*, fluconazole-resistant *Candida*, extended spectrum β-lactamase producing Enterobacteriaceae (ESBLs), vancomycin-resistant *Enterococcus* (VRE), multidrug-resistant *P. aeruginosa*, drug-resistant non-typhoidal *Salmonella*, drug-resistant *Salmonella typhi*, drug-resistant *Shigella*, methicillin-resistant *S. aureus* (MRSA), drug-resistant *Streptococcus pneumoniae*, and drug-resistant tuberculosis; in the category of concerning threats include vancomycin-resistant *S. aureus* (VRSA), erythromycin-resistant Group A *Streptococcus*, and clindamycin-resistant Group B *Streptococcus*.

The overuse and non-judicious use of antimicrobial agents have been among the leading factors of the development of antimicrobial resistance in bacteria. Moreover, the use of antibiotics as prophylaxis and growth promoters, lack of newly discovered antibiotics, and extensive use in food production animals also add to the development of this phenomenon. Nonetheless, antimicrobial resistance can also occur naturally as a result of exposure to antibiotics produced by antibiotic-producing microorganisms. Acquired resistance can be transferred both vertically and horizontally while intrinsic resistance can be transferred vertically. The development of antimicrobial resistance has contributed to the increased concern for public health issues. The resistant bacteria often are able to result in infections in both humans and animals. The emerging trend of antimicrobial resistance increases the frequency of changes and failures of treatments in both human and veterinary medicine (Nielsen *et al*., 2018; Ventola, 2015). As ownership of animals as pets and consumption of animal products have increased nowadays, the transfer of antimicrobial resistant bacteria from humans to animals or vice versa has been discovered and documented (Costa *et al*., 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Animal and sample collection

Forty carcasses (N=40) of wild-caught reticulated pythons were selected as samples using cross-sectional and opportunistic sampling technique at a snake abattoir located at Chaah, Segamat, Johor, Malaysia. All of them were wild-captured from an oil palm plantation situated at Changkat Jering, Perak, Malaysia. Out of the 40 sampled wild-caught reticulated pythons, there were 10 males and 30 females. A general physical examination was carried out on each of the selected reticulated python, and findings such as tick infestation were recorded on a form. The photos of their oral cavities were taken using a digital camera. The photos were used to evaluate the presence of stomatitis in the reticulated pythons. Oral swabs were obtained from the wild-caught reticulated pythons with or without lesions of stomatitis by using sterile transport swabs. The sterile transport swabs were stored in an ice box after sampling was done and during transportation to the laboratory.

3.2 Isolation and identification of bacteria

3.2.1 Primary culture

Each of the oral swab samples was inoculated on both the 7% blood and MacConkey agar plates. The oral swab was rolled onto one side of the agar, followed by streak plate method to obtain a well-isolated single colony in the primary culture. The inoculated blood and MacConkey agar plates were then incubated at 37°C for 24 hours under aerobic condition. After incubation, the plates were checked for bacterial growth. If there was no or little growth, the plates would again be incubated at 37°C for another 24 hours under aerobic condition and checked for growth after incubation. On the blood and MacConkey agar plates, the well isolated colonies were identified, and their morphology was described and recorded. The colony morphology was described according to the shape, size, colour, surface texture, haemolytic activity on blood agar, lactose fermentation on MacConkey agar, and smell.

For every well isolated colony on the blood and MacConkey agar plates, Gram staining was used to identify the cellular morphology of the bacteria. A portion of the colony was used to make a smear on a glass slide. The dry and heat-fixed smear was then flooded with Crystal violet for 1 minute. The glass slide was briefly rinsed with distilled water. After that, the glass slide was flooded with Lugol's iodine for 30 seconds. The glass slide was rinsed with distilled water. Acetone was used to decolourize the smear for about 3 seconds and rinsed with distilled water. The smear was then counter stained with diluted Carbol Fuchsin for 30 seconds and rinsed with distilled water. The glass slide was allowed to blot dry. By observing the glass slide using a light microscope under 1000x magnification with oil immersion, the Gram reaction, shape, arrangement of cells, and presence or absence of spores were recorded.

3.2.2 Pure culture

The colonies isolated from both the blood and MacConkey agar plates were inoculated onto nutrient agar plates using sterile inoculation loop and zigzag method as well as streak plate method. The inoculated nutrient agar plates were incubated at 37°C for 24 hours under aerobic condition. After incubation, the plates were checked for bacterial growth. If there was no or little growth, the plates would again be incubated at 37°C for another 24 hours under aerobic condition and checked for growth after incubation. For each colony obtained from the nutrient agar plates, their cellular morphology was determined by Gram staining. The cellular morphology was described according to the Gram reaction, shape, arrangement of cells, and presence or absence of spores.

3.2.3 Biochemical tests

3.2.3.1 Gram-positive aerobic bacteria

For gram-positive cocci, catalase test was used to differentiate *Staphylococcus* spp. from *Streptococcus* spp. To carry out a catalase test, one drop of hydrogen peroxide was placed onto a clean glass slide. A sterilized inoculation loop was used to take a portion of the bacterial colony. The colony was mixed thoroughly with the hydrogen peroxide. Bubble formation would be noted immediately for positive reaction, whereas bubble formation would be absent for negative reaction. *Staphylococcus* spp. would show positive reaction, while *Streptococcus* spp. would show negative reaction. *Staphylococcus* spp. produce catalase enzymes that hydrolyse hydrogen peroxide into water and oxygen; thus, bubble formation would be seen.

Coagulase test was carried out on the catalase positive cocci to differentiate the pathogenic from non-pathogenic species of *Staphylococcus*. A suspension was made by emulsifying a sufficient amount of colony in a drop of normal saline on a glass slide. A drop of rabbit serum was dropped onto the suspension. The mixture was stirred with a sterile inoculation loop and gently tilted back and forth. The fibrin clot would be observed within 10 seconds for positive reaction. The mixture of pathogenic species of *Staphylococcus* and rabbit serum would produce clumping. This is due to pathogenic *Staphylococcus* spp. produce coagulase enzyme that reacts with fibrinogen to form fibrin clot. Examples of coagulase-positive *Staphylococcus* include *S. aureus*, *S. pseudintermedius*, *S. intermedius*, *S. schleiferi*, *S. delphini*, *S. hyicus*, *S. lutrae*, and *S. hyicus*. Coagulase-negative *Staphylococcus* was subjected to AST using 0.04 Bacitracin to differentiate *Staphylococcus* from *Micrococcus*.

Staphylococcus would grow with no zone of inhibition; however, all *Micrococcus* would show zone ranges of 10.5 to 25 mm in diameter.

For gram-positive rods, absence of endospores and positive catalase reaction characterize the bacteria as *Corynebacterium* or *Actinomyces*. A few further tests were required to identify the genus and species of the bacteria. For instance, urease test, glucose test, nitrate reduction test, sucrose test, haemolysis test, trehalose test, motility test, and Christie-Atkins-Munch-Peterson (CAMP) test. On the other hand, presence of endospores could be divided into two main categories, which are non-motile and motile rods. If the rods are non-motile, *Bacillus anthracis* is identified, and it is encapsulated; if the rods are motile, *Bacillus* sp. is identified. *Bacillus cereus*, *Bacillus mycoides* and non-rhizoid variants are often non-motile. They are usually haemolytic, grow at 45 °C and ferment salicin.

3.2.3.2 Gram-negative aerobic bacteria

Spot oxidase test was used to identify the bacteria that produce cytochrome c oxidase. It is a rapid test that was done by applying bacterial isolates to filter papers moistened with 1% tetramethyl-p-phenylenediamine dihydrochloride. There would be a noticeable change of colour to blue or purple within 10 seconds in a positive oxidase test.

The identification of bacterial isolates was then proceeded with Triple Sugar Iron (TSI) test, sulfide-indole-motility (SIM) test, urease test, and citrate test. TSI test was done to determine the ability of an organism to ferment glucose, lactose, and sucrose, as well as their ability to produce hydrogen sulfide. The test is used primarily in the differentiation of members of the Enterobacteriaceae family from other gram-negative bacilli. It is also used to differentiate among Enterobacteriaceae based on their sugar fermentation patterns. The top of the well-isolated colony on the nutrient agar was touched with a sterile straight inoculating needle. The TSI agar was inoculated by first stabbing through the centre of the agar to the bottom of the tube and then streaking the surface of the agar slant. The butt was stabbed, and the slant was streaked. The inoculated tube was capped loosely and incubated at 37°C for 24 hours. Observations were made and recorded after incubating the tube. Table 3.1 shows the expected results of the test.

Table 3.1: Interpretation of TSI test

Results	Interpretation
Alkaline/acid (red slant/yellow butt) reaction	Glucose fermentation only, peptone catabolized
Acid/acid (yellow slant/yellow butt) reaction	Fermentation of glucose, lactose and/or sucrose
Alkaline/alkaline (red slant, red butt) reaction	Absence of carbohydrate fermentation, peptone catabolized under aerobic and/or anaerobic conditions.
Blackening of the agar	Presence of hydrogen sulfide
Bubbles or cracks in the agar	Gas production (formation of carbon dioxide and hydrogen gas)

The bacterial isolates were further identified using SIM test. This test was used to determine the ability of an organism to reduce sulfur-containing compounds to sulfides to produce hydrogen sulfide, and to decompose the amino acid tryptophan to indole. Differentiation between motile and non-motile organisms can also be done by this test. The organism was inoculated into the SIM agar by means of stab inoculation. The inoculated tube was incubated at 37°C for 24 hours. After incubation, about 0.5 ml of Kovac's reagent was added to the tube to perform indole test. The results were observed and recorded. The interpretation of the results is summarized in Table 3.2.

Table 3.2: Interpretation of SIM test

Tests	Results	Interpretation
Sulfide test	Blackening on the agar	Positive result
	No blackening on the agar	Negative result
Indole test	Formation of a pink to red colour ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent	Positive result
	No formation of a pink to red colour ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent	Negative result
Motility test	Diffuse, hazy growths that spread throughout the agar rendering it slightly opaque	Positive result
	Growth that is confined to the stab-line, with sharply defined margins and leaving the surrounding agar clearly transparent	Negative result

Moreover, urease test was done to identify the bacterial isolates, too. It is used to determine the ability of an organism to hydrolyse urea to produce ammonia and carbon dioxide, through the production of urease enzyme. The slant of the Christensen's Urea Agar was inoculated with a portion of a colony. The tube was capped loosely and incubated at 37°C for 24 hours, followed by observing for and recording any colour change. A positive reaction is indicated by a pink colour, while a negative reaction is indicated by no colour change.

Furthermore, citrate test is included in the biochemical tests for characterization of the bacterial isolates. It is used to test the ability of an organism to utilize citrate as a source of energy. The slant of the Simmon's Citrate Agar was streaked with a light inoculum picked from a colony. The tube was capped loosely, incubated at 37°C for 24 hours, as well as observed and recorded for any colour change after incubation. A colour change from green to blue along the slant indicates positive reaction, whereas no colour change indicates negative reaction.

Other than the aforementioned tests, additional tests were carried out in the process of identifying the bacterial isolates. The decisions to which additional tests were required were based on the guidance provided in a book entitled "A Diagnostic Manual of Veterinary Clinical Bacteriology and Mycology" written by Jang *et al.* in 2010. Table 3.3 depicts the additional tests used and their objectives and procedures, while Table 3.4 shows their expected results and interpretation.

Table 3.3: Additional tests and their respective objectives and procedures

No	Tests	Objectives	Procedures
1.	Hanging drop	To determine the motility of an organism if the organism is non-motile on SIM test, and TSI result shows alkaline/alkaline reaction.	The inoculum was prepared by mixing a portion of the colony with approximately 1 ml of distilled water. A loopful of the inoculum was placed on a glass slide using a sterile inoculating loop. The glass slide was viewed using a dark field microscope under

			x20 objectives. Motility of the organism was observed and recorded.
2.	Phenylalanine deaminase (PD) test	To test the ability of an organism to produce the enzyme deaminase.	The phenylalanine slant was inoculated with a portion of a colony. The tube was incubated at 37°C for 24 hours. Four to five drops of 10% aqueous ferric chloride ($FeCl_3$) solution were added to the slant. Then, the tube was rotated to dislodge the surface colonies. The colour change was observed and recorded.
3.	Oxidation-Fermentation (OF) test	To determine the oxidation or fermentation of carbohydrates by bacteria.	The OF media were inoculated in duplicate for each test colony by means of stabbing. One of the media was added with sterile mineral oil. The tubes were covered and incubated at 37°C for 24 hours. The colour change was observed and recorded.

4.	Lysine Decarboxylase (LDC) test	To determine the ability of an organism to use the amino acid lysine as a source of carbon and energy for growth. Use of lysine is accomplished by the enzyme lysine decarboxylase.	The colony was touched and inoculated to the LDC broth using a sterile inoculating loop. The tube was added with 4 to 5 drops of sterile mineral oil, covered, and incubated at 37°C for 24 hours. The colour change was observed and recorded.
5.	Ornithine Decarboxylase (ODC) test	To determine the ability of an organism to use the amino acid ornithine as a source of carbon and energy for growth. The use of ornithine is accomplished by the enzyme ornithine decarboxylase.	The colony was touched and inoculated to the ODC broth using a sterile inoculating loop. The tube was added with 4 to 5 drops of sterile mineral oil, covered, and incubated at 37°C for 24 hours. The colour change was observed and recorded.
6.	O-Nitrophenyl-β-D-galactopyranoside (ONPG) test	To determine the ability of an organism to produce the enzyme β-galactosidase that hydrolyses the substrate ONPG to form a visible (yellow) product, orthonitrophenol. To	An ONPG disk was placed into a sterile tube and 0.2 ml of saline was added. A loopful of the test colony was inoculated in the tube. The tube was added with 4 to 5 drops of sterile mineral oil, covered,

		distinguish late lactose fermenters from non–lactose fermenters of Enterobacteriaceae.	and incubated at 37°C for up to 4 hours. Colour change of the disk was observed and recorded.
7.	Polyvalent 'O' antisera test	To identify if an organism belongs to the genus *Salmonella*.	A suspension was made by emulsifying enough colony in a drop of normal saline on a glass slide. A drop of polyvalent 'O' antisera was dropped onto the suspension. The mixture was stirred with a sterile inoculation loop and gently rocked. The presence or absence of agglutination was observed and recorded.
8.	Polymyxin B	To differentiate between Enterobacter spp./Pantoea and Serratia spp.	AST was done by using Kirby-Bauer disk diffusion method and polymyxin B as the antimicrobial agent to be tested. The zone of inhibition was measured following incubation of the inoculated Mueller-Hinton agar at 37°C for 24 hours.

Table 3.4: Additional tests and their respective expected results and interpretation

No	Tests	Expected results	Interpretation
1.	Hanging drop	The organisms move in different directions and change their positions in the field	Motile
		The organisms jiggle at one place	Non-motile
2.	PD test	A colour change from straw/yellow colour to green colour	Positive reaction
		No colour change	Negative reaction
3.	OF test	Development of a yellow colouration in the open tube only	Positive oxidative reaction (O^+/F^-)
		Development of a yellow colouration in both open and closed tubes	Positive fermentative reaction (O^+/F^+)
		No colour change	Non-oxidizer/Non-fermenter (O^-/F^-)
4.	LDC test	A colour change from yellow to purple	Positive reaction
		No colour change	Negative reaction
5.	ODC test	A colour change from yellow to purple	Positive reaction
		No colour change	Negative reaction
6.	ONPG test	Yellow colouration	Positive reaction

		Colourless	Negative reaction
7.	Polyvalent 'O' antisera test	Presence of agglutination	The organism belongs to the genus *Salmonella*
		Absence of agglutination	The organism does not belong to the genus *Salmonella*
8.	Polymyxin B	Zone of inhibition of more than 11 mm	Susceptible to polymyxin B
		Zone of inhibition of less than or equals 11 mm	Resistant to polymyxin B

3.3 Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method was used to identify the antimicrobial susceptibility or resistance of the bacteria to various selected antimicrobial agents. Gentamicin (10 µg), amoxicillin (10 µg), cephalexin (30 µg), azithromycin (15 µg), tetracycline (30 µg), and ciprofloxacin (5 µg) were chosen as the antimicrobial agents to be tested for the antimicrobial susceptibility among the bacterial isolates which had been identified. For every identified bacterial isolate, four or five well-isolated colonies were touched using a sterile inoculating loop. The colonies were suspended in 2 ml of sterile distilled water which had been poured into a sterile test tube. A smooth suspension of inoculum was created. The turbidity of the suspension was visually compared to a 0.5 McFarland standard by using a Wickerham card as the background. The suspension with a comparable turbidity with the standard was used as an inoculum. In cases where the turbidity was different from the standard, adjustment was done by either diluting with sterile distilled water if the suspension was more turbid than the standard or adding more colonies if the suspension was less turbid than the standard.

A sterile cotton swab was dipped into the prepared inoculum. The swab was then rotated with a firm pressure against the inside wall of the tube above the liquid level to remove excess fluid. The Mueller-Hinton agar was inoculated using the dipped swab by streaking method. Streaking was done three times over the same agar, each time with the agar being streaked in one direction and rotated 90 degrees after the previous streaking was done. The agar plate was then rimmed to remove excess fluid and allowed to dry for approximately 5 minutes at room temperature.

By using an antibiotic disc dispenser, six selected antibiotic discs were dispensed onto the inoculated Mueller-Hinton agar. The dispensed discs were pressed gently to the agar using a pair of sterile forceps to ensure that they were well-attached. After that, the agar plate was incubated at 37°C for 24 hours. After incubation, the zone of inhibition which is the area without growth around the antibiotic disc was measured by using a digital caliper. The measurement was done for every dispensed antibiotic disc and recorded in millimetres. The results were interpreted as resistant, intermediate, or susceptible by referring to the tables provided in the Clinical and Laboratory Standards Institute (CLSI) AST standards. CLSI documents VET01-S2 and M100-S20 were the ones used in the interpretation of the measurements of zone of inhibition.

3.4 Statistical analyses

A descriptive analysis was used to obtain the prevalence of stomatitis in the wild-caught reticulated pythons. A chi-square test was used to analyse the relationship between the occurrence of stomatitis with sex and ectoparasitic infestation. The analysis was aimed to determine the association of these two findings with the occurrence of bacterial stomatitis in the wild reticulated pythons. The association between the occurrence of stomatitis and the identified corresponding bacterial isolates was also analysed using a chi-square test. Both the tests were analysed at a significance level of $p < 0.05$.

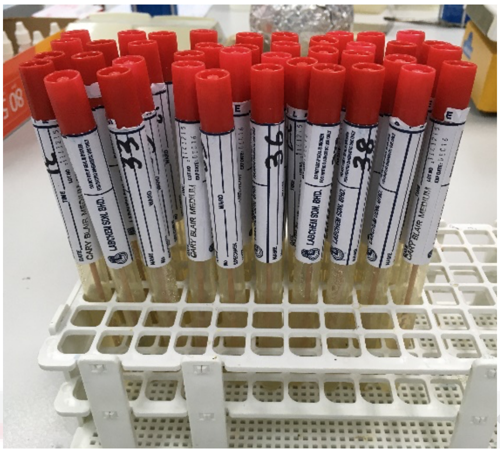

Figure 3.1: Samples collected into the transport media

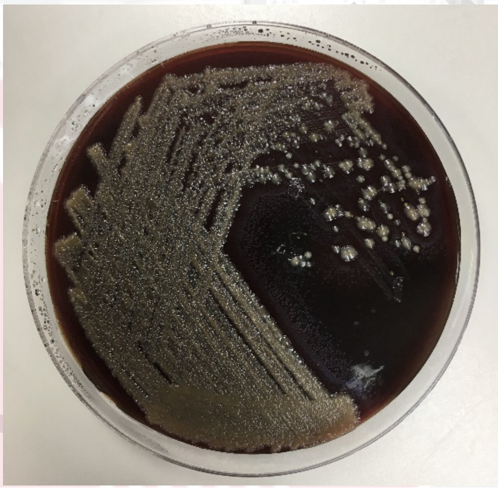

Figure 3.2: Bacterial isolation on blood agar

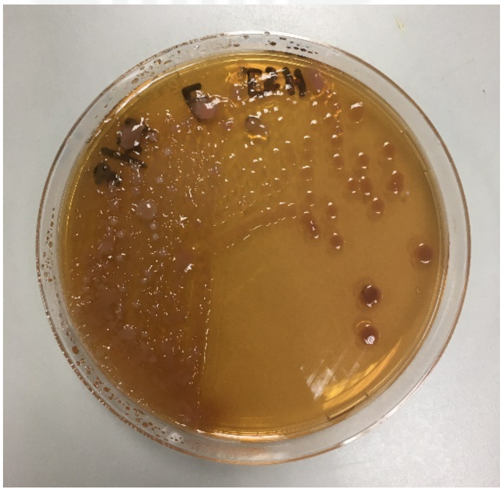

Figure 3.3: Bacterial isolation on MacConkey agar

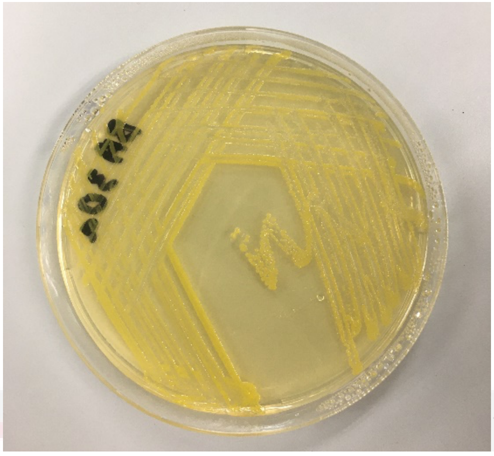

Figure 3.4: Bacterial isolation on nutrient agar

Figure 3.5: Antimicrobial susceptibility testing

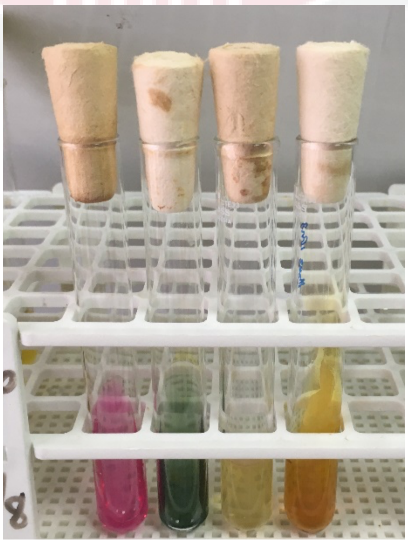

Figure 3.6: Biochemical tests (from left to right: urease test, citrate test, SIM test, TSI test)

CHAPTER 4

RESULTS

4.1 Bacterial stomatitis

The prevalence of stomatitis in the sampled wild-caught reticulated pythons is 77.5%. The prevalence of the males and females to have stomatitis were 100% (10/10) and 70.0% (21/30) respectively. There was a significant difference of $p < 0.05$ between the occurrence of stomatitis in males and females. Among the reticulated pythons with tick infestation (60.0%, 24/40), 79.2% of them suffered from stomatitis, whereas 75.0% of the reticulated pythons without tick infestation suffered from stomatitis. The occurrence of stomatitis was comparable ($p > 0.05$) in the reticulated pythons with or without tick infestation.

4.2 Bacterial isolates

A total of 153 bacterial isolates were identified. There were 24 bacterial species from 18 genera among these bacterial isolates. Out of the 24 bacterial species, only two (8.3%) were gram-positive aerobic bacteria, with the others (91.7%) being gram-negative aerobic bacteria. The most predominant bacterial isolates in the oral cavities of the reticulated pythons were *Aeromonas* sp. (14.4%), followed by *K. pneumoniae* (11.8%), and *Alcaligenes faecalis* (8.5%). Table 4.1 and Figure 4.1 show the prevalence of bacteria in the oral cavities of the reticulated pythons.

Table 4.1: Prevalence of bacteria in the oral cavities of the reticulated pythons arranged in alphabetical order

Bacteria	Number
Acinetobacter baumanii	9 (5.9%)
Acinetobacter lwofii	3 (2.0%)
Aeromonas sp.	22 (14.4%)
Alcaligenes faecalis	13 (8.5%)
Bordetella bronchiseptica	6 (3.9%)
Citrobacter freundii	4 (2.6%)
Citrobacter sp.	2 (1.3%)
Corynebacterium sp.	1 (0.7%)
Coagulase-negative *Staphylococcus*	7 (4.6%)
Escherichia coli	4 (2.6%)
Enterobacter aerogenes	1 (0.7%)
Enterobacter cloacae	8 (5.2%)
Enterobacter spp.	8 (5.2%)
Klebsiella oxytoca	10 (6.5%)
Klebsiella pneumoniae	18 (11.8%)
Klebsiella sp.	6 (3.9%)
Plesiomonas shigelloides	3 (2.0%)
Proteus spp.	10 (6.5%)
Pseudomonas aeruginosa	3 (2.0%)
Salmonella sp.	3 (2.0%)
Serratia spp.	4 (2.6%)
Stenotrophomonas maltophilia	2 (1.3%)
Yersinia pestis	3 (2.0%)
Vibrio cholerae-01	3 (2.0%)

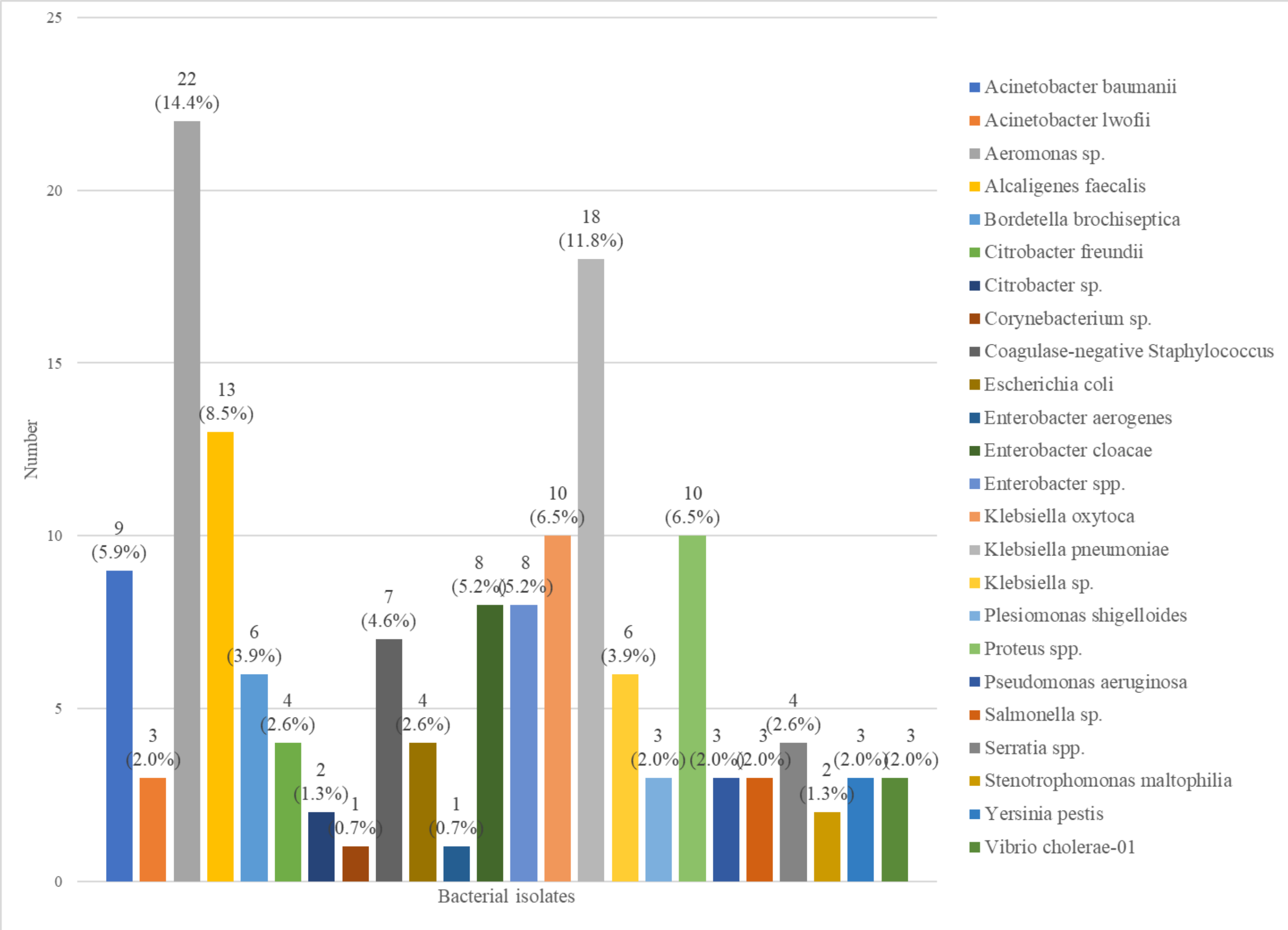

Figure 4.1: Prevalence of bacteria in the oral cavities of the reticulated pythons

In the oral cavities of the reticulated pythons with lesions of stomatitis, 5.1% (6/117) of the bacterial isolates were gram-positive aerobes and 94.9% (111/117) of the bacterial isolates were gram-negative aerobes as shown in Figure 4.2. Figure 4.3 shows that in the oral cavities of the reticulated pythons without lesions of stomatitis, 5.6% (2/36) of the bacterial isolates were gram-positive aerobes and 94.5% (34/36) of the bacterial isolates were gram-negative aerobes.

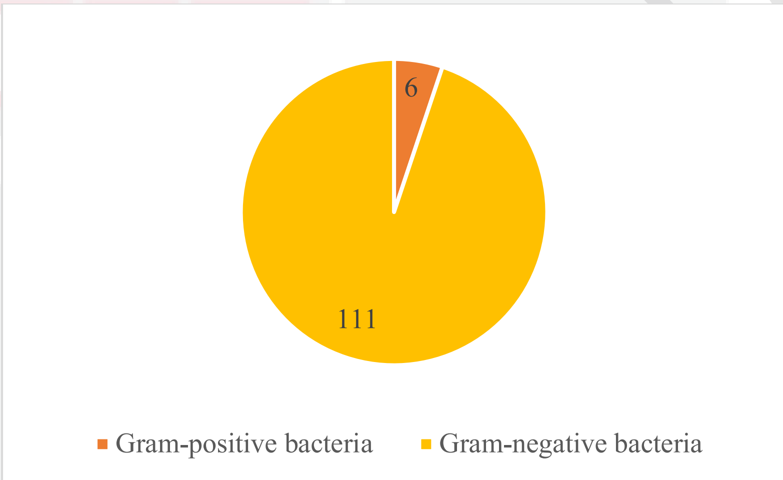

Figure 4.2: Proportion of bacteria in the oral cavities of the reticulated pythons with stomatitis

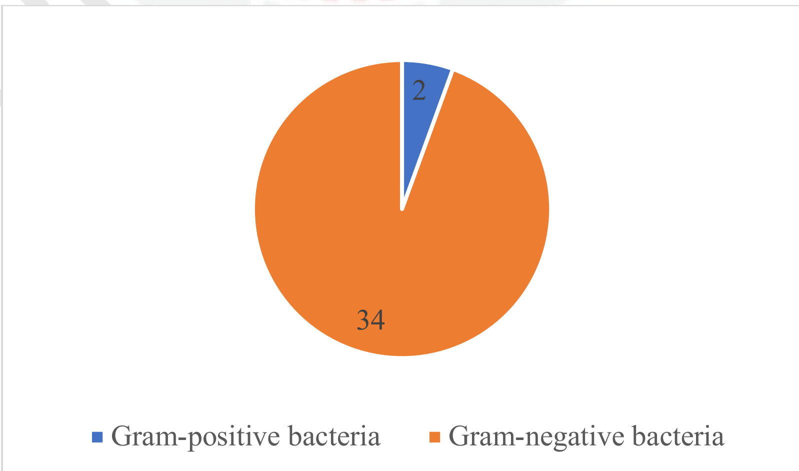

Figure 4.3: Proportion of bacteria in the oral cavities of the reticulated pythons without stomatitis

Most of the reticulated pythons with or without stomatitis had polymicrobial cultures in their oral cavities. Only 2.5% (1/40) of the python had a single bacterial species being isolated which was also presented with stomatitis. The number of oral bacterial isolates ranged from two to six from a single python. Two bacterial isolates were identified from 3 pythons (7.5%), three isolates from 10 pythons (25.0%), four isolates from 17 pythons (42.5%), five isolates from 6 pythons (15.0%), and six isolates from 3 pythons (7.5%). *Corynebacterium* sp. (0.7%), *Enterobacter aerogenes* (0.7%), *Klebsiella oxytoca* (6.5%), *Salmonella* sp. (2.0%), and *S. maltophilia* (1.3%) were only isolated from the oral cavities of reticulated pythons with lesions of stomatitis being present. There was an association between the presence of *K. oxytoca* in the oral cavity and the occurrence of stomatitis ($p < 0.05$).

4.3 Antimicrobial susceptibility testing

Table 4.2 shows the results of AST performed on the identified bacterial isolates. Table 4.3 depicts the percentages of antimicrobial susceptibility and resistance of the antimicrobial agents tested.

Table 4.2: Results of AST

Bacteria	Gentamicin	Amoxicillin	Cephalexin	Azithromycin	Tetracycline	Ciprofloxacin
Acinetobacter baumanii	S	R	R	I	S	S
Acinetobacter lwofii	S	R	R	R	S	S
Aeromonas sp.	S	R	R	I	S	S
Alcaligenes faecalis	S	R	R	R	S	S
Bordetella bronchiseptica	S	R	R	I	S	S

Citrobacter freundii	S	R	R	R	S	S
Citrobacter sp.	S	R	R	I	S	S
Corynebacterium sp.	R	R	R	R	R	R
Coagulase-negative *Staphylococcus*	S	S	I	R	S	S
Escherichia coli	S	R	S	R	S	S
Enterobacter aerogenes	S	R	R	R	S	S
Enterobacter cloacae	S	R	R	R	R	S
Enterobacter spp.	S	R	R	I	S	S
Klebsiella oxytoca	S	R	S	R	S	S
Klebsiella pneumoniae	S	R	S	R	S	S
Klebsiella sp.	S	R	R	I	S	S
Plesiomonas shigelloides	S	R	I	I	S	S
Proteus spp.	S	S	R	R	R	S
Pseudomonas aeruginosa	S	R	R	R	S	S
Salmonella sp.	S	R	R	I	S	S
Serratia spp.	S	R	R	R	R	S
Stenotrophomonas maltophilia	S	S	R	S	S	S
Yersinia pestis	S	S	S	I	S	S
Vibrio cholerae-01	S	R	R	I	R	S

Note: S = Susceptible

I = Intermediate

R = Resistant

Table 4.3: Percentages of antimicrobial susceptibility and resistance of the tested antimicrobial agents

	Gentamicin	Amoxicillin	Cephalexin	Azithromycin	Tetracycline	Ciprofloxacin
Susceptible	23 (95.8%)	4 (16.7%)	4 (16.7%)	1 (4.2%)	19 (79.2%)	23 (95.8%)
Intermediate	0	0	2 (8.3%)	10 (41.7%)	0	0
Resistant	1 (4.2%)	20 (83.3%)	18 (75.0%)	13 (54.2%)	5 (20.8%)	1 (4.2%)

Most of the bacterial species were equally susceptible to gentamicin and ciprofloxacin, with both the percentages being 95.8%. Besides, some bacterial isolates were resistant to tetracycline (20.8%). Twenty (83.3%) and eighteen (75.0%) bacterial species were reported to be resistant to amoxicillin and cephalexin, respectively, showing a high resistance profile. Azithromycin was the antimicrobial agent that showed the most intermediate result of the test, recording ten (41.7%) bacterial species to have that result, followed by cephalexin (8.3%). *Corynebacterium* sp. was resistant to all the antimicrobial agents tested. A high proportion (75.0%; 18/24) of the bacterial isolates tested was multi-drug resistant (MDR), demonstrating antimicrobial resistance to at least one antimicrobial agent in three or more antimicrobial categories. Coagulase-negative *Staphylococcus*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *S. maltophilia*, and *Yersinia pestis* were among the ones which were not MDR.

CHAPTER 5

DISCUSSION

In this study, a high proportion of the wild-caught reticulated pythons were presented with stomatitis, accounting for 77.5% in all the pythons. This agrees with the description of infectious stomatitis by Mehler and Bennett (2006) as they state that it is the most common clinical form of upper alimentary tract disease in reptiles. The high occurrence of stomatitis in these pythons could be attributed to the wild environment of their habitats. Environment of poor quality, traumatic injury, or bite wounds are among the factors of the development of stomatitis in reptiles (Jho *et al.*, 2011b). The pythons could get injured when they compete for food with other animals or when they prey. Male pythons are believed to involve themselves more in fighting for dominance or mates; thus, the prevalence of stomatitis in male pythons is higher than that in female pythons. Inconducive wild environment could lead to stress in the pythons, causing them to be immunocompromised and more prone to developing diseases.

Gram-negative aerobic bacteria were mainly isolated in this study. This is comparable to the findings of the aerobes found in the oral cavities of Lancehead snakes (*Bothrops atrox*) with evidence of stomatitis (Pereira *et al*., 2017). The predominant gram-negative aerobes were *Aeromonas* sp. and *K. pneumoniae*. A previous study claimed that *Aeromonas* sp. was revealed to be the most isolated organism from the oral cavity of snakes, followed by *Pseudomonas* spp., *Proteus* spp., and *E. coli* (Cooper & Leakey, 1976). However, a more recent study was conducted by Yak *et al*. in 2015 to detect the bacterial microflora of the oral cavities of free-living reticulated pythons in Singapore. The results showed that the most commonly identified bacterial species was *Pseudomonas* spp., followed by *Staphylococcus* *sciuri*. Interestingly, *S. sciuri* was not isolated in the oral cavities of the reticulated pythons in this study.

Another study also found out that *Pseudomonas* sp. had the highest incidence rate of the bacteria isolated from the oral cavity of snakes (Jho *et al*., 2011b). Although coagulase-negative *Staphylococcus* was only accounted for 4.6% among all the identified bacterial isolates, it was the most isolated bacteria in a study conducted in Iran by Dehghani *et al.* (2015), with the percentage of 34.5% while *Pseudomonas* (3.1%) was the least isolated bacteria. The findings of the oral bacteria from snakes were surprisingly variable in different studies. There was an absence of a noticeable trend of the specific bacterial species isolated from the oral cavity of snakes. This can possibly be attributed to the differences in the snakes from the aspects of the geographical locations of habitats, strategies of predation, and type of animals they preyed on (Shek *et al*., 2009). Nevertheless, gram-negative isolates still predominated in most previous studies (Lukač *et al*., 2017; Dipineto, 2014; Lam *et al*., 2010; Blaylock, 2001) which is similar to the findings of this study. Additionally, a high proportion of the bacteria isolated from the oral cavities of the reticulated pythons sampled for this study is in the family of Enterobacteriaceae. The feeding behaviours of snakes could possibly be a reason of this phenomenon. The snakes would first eat the head of prey, leaving the faecal flora of the prey to colonize in the oral cavity (Goldstein *et al*., 1979).

Among the thirty-one reticulated pythons presented with stomatitis in this study, thirty (96.8%) of them had a mixed infection of bacterial stomatitis. This is in line with the findings in a study conducted by Pereira *et al.* in 2007. Majority of the bacterial isolates in this study are considered as part of the normal flora of the oral cavity of snakes (Artavia-León *et al*., 2017; Jho *et al*., 2011a). However, many of these bacteria can serve as opportunistic pathogens that are able to result in clinical diseases by invading the visceral organs when the snake itself as a host is immunocompromised (Mader, 2005). Besides, they also pose a public health concern as numerous of them are zoonotic bacteria which can cause infections in humans. One of the ways of humans acquiring bacteria from snakes is through snakebite. Following a snakebite, there is a high chance that the wound would become infected, and multiple bacteria could be commonly isolated (Artavia-León *et al*., 2017; Yak *et al*., 2015).

In this study, *Aeromonas* sp. was the most isolated organism. *A. hydrophila* is known to cause severe infections in humans after snakebites which can be fatal (Mukhopadhyay *et al*., 2008). It can also lead to death in snakes due to bacteraemia (Orozova *et al*., 2012). Moreover, it is capable of resulting in diarrhoea and infection of soft tissue following minor trauma being exposed to fresh water containing the organism. *Salmonella* sp. is an important bacterial species as it has a wide host range and can cause diseases in both humans and animals. Hardy (2004) states that *Salmonella* had been an unresolved problem for over a hundred years in public health, epidemiology, and microbiology. It had been described by Sting *et al.* (2013) that it is an opportunistic organism in immunocompromised lizards and snakes. Due to the increased ownership of exotic pets in the present society, the issue of salmonellosis should be emphasized. Reptiles as exotic pets do harbour *Salmonella* and shed the organism in their faeces, and various *Salmonella* serotypes had been identified from these reptilian pets; therefore, humans with immature or poor immune system are advised to refrain from having contact with reptiles (David *et al.*, 1997).

Pathogenic strains of *E. coli* can cause intestinal and extra intestinal diseases in both humans and animals, such as diarrhoea, cystitis, and meningitis (Ramos *et al.*, 2019). Wild animals can serve as a reservoir of pathogenic strains of *E. coli* after their intestinal microbiota has a change in the populations of *E. coli* due to living closely with humans (Iovine *et al.*, 2015). *P. aeruginosa* has been known to cause skin and soft tissue infections in humans such as folliculitis, ecthyma gangrenosa in neutropenic patients, and in burn wounds (Pier & Ramphal, 2005). *A. faecalis* was found to be the third most common bacterial isolate from the oral cavities of the pythons. It has the ability to cause skin and soft tissue infections in humans, too (Tena *et al.*, 2015). Besides, the second most isolated organism was *K. pneumoniae*. It is considered to be an important nosocomial agent which can lead to pneumonia in patients associated with alcoholism or diabetes mellitus, and urinary tract infections in humans (Ashurst & Dawson, 2020; Marques *et al.*, 2019).

Y. pestis was isolated in this study as well. It can cause plague primarily in rodents and can be transmitted by fleas that carry the organism from the infected wild rodents to humans, resulting in bubonic plague (Falcão, 2014). The presence of this organism could possibly be due to ingestion of the infected rats before the pythons were captured since the pythons were caught from wild habitat. However, more studies are needed in the future to validate this statement. *Vibrio cholerae-01* is the causative agent of cholera that thrives in aquatic habitats. Humans and animals can acquire this organism through water sources contaminated by the faecal materials from the infected individuals (Laviad-Shitrit *et al.*, 2019). In relation to this, the reticulated python naturally is an excellent swimmer, in which it could acquire the organism while it is in the water.

In this study, *S. maltophilia* was isolated from only two pythons, one with stomatitis while the other one without stomatitis. Although this organism is part of the normal flora of the oral cavity of snakes, it was described to be involved in cases of ulcerative stomatitis in snakes (Hejnar *et al*., 2007). It is usually a nosocomial infection in humans and can lead to diseases such as pneumonia, blood-stream infection, wound, and urinary tract infection (Looney *et al.*, 2009). Coagulase-negative *Staphylococcus* is the most isolated gram-positive aerobes in this study. Yak *et al.* (2015) state that coagulase-negative *Staphylococcus* is a common organism in the oral cavity of snakes, and it can cause infections in humans. Coagulase-negative *Staphylococcus* is also among the common bacterial isolates from infected wounds due to snakebites in humans (Garg *et al.*, 2009).

The results of AST surprisingly demonstrated that most of the bacteria isolated from this study were MDR organisms, accounting for 75.0% of the total isolates. This is a worrying issue for both animal and human health. The high incidence of antimicrobial resistance in the wild reticulated pythons which had not been apparently exposed to antibiotics could be due to the environment that they inhabited. The role of the environment in the spread of antimicrobial resistance has been well-known (Prestinaci *et al.*, 2015). The environment might have been contaminated with bacteria that carry the resistance genes as well as antimicrobial residues. Antimicrobial resistant organisms and antimicrobial residues could still be present in the sewage from human neighbourhoods and animal production industry, even if the sewage had been treated in wastewater treatment plants (Costa *et al.*, 2013). The sewage system might drain to the wild environment, causing it to be contaminated with the resistant bacteria and antimicrobial residues. The antimicrobial residues are usually of sub-inhibitory concentrations, in which the abundance of microbiota in the environment can interact with, eventually resulting in the formation of antimicrobial resistant organisms (Costa *et al.*, 2013).

Amoxicillin and cephalexin were shown to be ineffective against the bacterial isolates from the oral cavities of reticulated pythons in this study as the commonly used first-line antibiotics. The resistance rates were recorded high for both amoxicillin (83.3%) and cephalexin (75.0%). The results for amoxicillin are comparable to the results in a study conducted by Lam *et al.* in 2010. Majority of the isolates were sensitive ciprofloxacin and gentamicin, accounting for 95.8% in both of them. This agrees with the results of a study conducted by Garg *et al.* in 2009. In the treatment of bacterial stomatitis, fluoroquinolones and aminoglycosides are the common choices of first-line antibiotics while waiting for the results of bacterial culture and AST (Mehler & Bennett, 2006). Therefore, ciprofloxacin and gentamicin could be the first-line antibiotics in treating bacterial stomatitis or even other bacterial infections in the reticulated pythons or other species of snakes. These antibiotics would also be a good option of antibiotics to be used in the cases of snakebite or other snake-related bacterial diseases in humans.

CHAPTER 6

CONCLUSION

In this study, the wild-caught reticulated pythons were highly prevalent for bacterial stomatitis of polymicrobial nature. The bacterial population in the oral cavities of pythons with or without stomatitis was predominated by gram-negative bacteria, with *Aeromonas* sp. being the most isolated organism. Therefore, bacterial stomatitis in the wild-caught reticulated pythons is usually an infection caused by multiple agents rather than a single agent. Majority of the bacterial isolates tested were MDR, with the exceptions of coagulase-negative *Staphylococcus*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *S. maltophilia*, and *Y. pestis*. Nevertheless, gentamicin and ciprofloxacin are still the reasonable empirical choice of antibiotics. In contrary, the use of amoxicillin and cephalexin should be avoided as they would not provide any therapeutic benefit.

CHAPTER 7

RECOMMENDATIONS

An aerobic condition was used for the isolation of oral bacteria from the wild-caught reticulated pythons; as a result, only the aerobic bacteria were isolated in this study. In fact, the anaerobic bacteria could also serve as the important causative agents of bacterial stomatitis in the pythons. Therefore, isolation of the anaerobic bacteria is recommended in future studies. Apart from that, stomatitis in the reticulated pythons could also be resulted from aetiological agents other than bacteria, which are viruses, fungi, or parasites. Thus, determination of the role of these agents in the occurrence of stomatitis in pythons is crucial and recommended as well.

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