UNIVERSITI PUTRA MALAYSIA

ISOLATION AND IDENTIFICATION OF NORMAL FLORA FROM THE CLOACA OF MALAYAN BOX TURTLES

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ISOLATION AND IDENTIFICATION OF NORMAL FLORA FROM THE CLOACA OF MALAYAN BOX TURTLES

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Isolation and Identification of Normal Flora from Cloaca of Malayan Box Turtles", by Syadatul Akma binti Raidi 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 – Project

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DEDICATIONS

This project paper is dedicated to the Allah SWT, who made all things possible,

To my family,

Father

Mother

Brother, Sisters

And to all my teachers who have committed themselves towards the noble cause of education.

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It is with deepest appreciation and gratitude that I thank God and all those who have made this project paper a reality.

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
H_2O_2	Hydrogen peroxide
CO_2	Carbon dioxide
H_2S	Hydrogen sulphide
mm	Milimetre
MR/VP	Methylene Red/Vogeus-Prokeur
IUCN	International Union for Conservation of Nature and Natural
CITES	Convention on International Trade in Endangered Species
CONS	Coagulase Negative *Staphylococcus*
CDC	Centers for Disease Control and Prevention
OEPP	European and Mediterranean Plant Protection Organization

ABSTRAK

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

PENGASINGAN DAN PENGENALPASTIAN FLORA NORMAL DARIPADA KLOAKA KURA-KURA KATUP MALAYA

Oleh

Syadatul Akma binti Raidi

2016

Supervisor: Dr. Hazilawati binti Hamzah

Co-supervisor:

Prof. Abdul Rani bin Bahaman

Kura-kura Katup Malaya, *Cuora amboinensis kamaroma* adalah reptilia, daripada order Testudines, dibawah famili Geoemydidae dan subfamili Geoemydinae. Spesies ini disenaraikan sebagai spesies yang terpengaruh di Malaysia di bawah Senarai Merah Spesies Terancam oleh Kesatuan Kebangsaan untuk Pemuliharaan Alam Semulajadi dan Sumber Semulajadi. Tujuan kajian ini adalah untuk menentukan kehadiran bakteria di dalam kloaka Kura-kura Katup Malaya. Sejumlah 8 sampel kloaka telah dikumpul daripada kura-kura katup dewasa yang sihat dengan menggunakan swab steril. Pengasingan dan pengenalpastian telah dilakukan untuk menentukan

spesies bakteria. Sembilan spesies bakteria telah dapat diasingkan dan dikenalpasti daripada sampel. Lapan spesies bakteria Gram negatif dan satu spesies Gram positif bakteria yang telah dapat diasingkan dan dikenalpasti termasuklah *Staphylococcus aureus, Acinetobacter lwoffi, Escherichia coli, Pasteurella testudinis, Pantoea agglomerans, Acinetobacter calcoaticus, Salmonella* spp., *Klebsiella oxytoca* dan *Serratia* sp.. Kebanyakan bakteria yang dapat diasingkan adalah flora normal kecuali *S. aureus, E. coli, Salmonella spp*., *K. oxytoca* dan *Serratia sp.* yang merupakan zoonotik. Mikroorganisma ini boleh menyebabkan jangkitan yang teruk terutamanya kepada mereka yang sakit atau mempunyai daya imun yang lemah. Kesimpulannya, , langkah berjaga-jaga yang sesuai perlu diambil semasa pengendalian dan pengangkutan haiwan untuk menghalang mana-mana penghantaran bakteria zoonotik dari haiwan kepada manusia.

Kata kunci: Kura-kura Katup Malaya, kloaka, zoonosis, flora normal, reptilia

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Final Year Project

ISOLATION AND IDENTIFICATION OF NORMAL FLORA OF THE CLOACA OF THE MALAYAN BOX TURTLES

By

Syadatul Akma binti Raidi

2016

Supervisor: Dr. Hazilawati binti Hamzah

Co-supervisor:

Prof. Abdul Rani bin Bahaman

Malayan Box turtle, *Cuora amboinensis kamaroma* is a reptile, of the order Testudines, under the family of Geoemydidae and subfamily Geoemydinae. This species is listed by the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species as a vulnerable species in Malaysia. The aim of this study is to determine the presence of bacteria in the cloaca of Malayan Box Turtle. A total of 8 cloacal samples were collected from adult, healthy Malayan Box turtles by using sterile swabs. Bacteria isolation and

identification were done to determine species of the bacteria. Nine species of bacteria were isolated and identified from the samples. Only one species of Gram positive and 8 species of Gram negative bacteria were isolated and identified which include *Staphylococcus aureus, Acinetobacter lwoffi, Escherichia coli, Pasteurella testudinis, Pantoea agglomerans, Acinetobacter calcoaticus, Salmonella* spp., *Klebsiella oxytoca* and *Serratia* sp.. Most of the isolated bacteria were normal flora except for *S. aureus, E. coli, Salmonella spp*., *K. oxytoca* and *Serratia sp.* which are zoonotic. These microorganisms can cause severe infection especially in people who are sick or immunocompromised. In conclusion, appropriate hygienic precautions need to be taken during handling and transporting of the animal to prevent any transmission of zoonotic bacteria from the animal to human.

Keywords: Malayan Box turtle, cloaca, zoonosis, normal flora, reptile

1.0 INTRODUCTION

1.1 Malayan Box Turtle, *Cuora amboinensis kamaroma*

Malayan Box turtle, *Cuora amboinensis kamaroma* is under the family of Geoemydidae and subfamily Geoemydinae (Spinks *et al*., 2004). They are a small (straight carapace length to 25 cm), semi-aquatic turtle, largely restricted to standing water bodies of Southeast Asia, from eastern India to Indonesia and the Philippines (Schoppe, 2011). Under The International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species, it is considered as endangered in Bangladesh, Cambodia, Lao, Viet Nam, and vulnerable in India, Indonesia, Malaysia, Thailand (not listed by European and Mediterranean Plant Protection Organization [OEPP] 1997) and no information available for Myanmar; presumed stable in Singapore (small population). Under the species of *Cuora amboinensis* asides from *Cuora amboinensis kamaroma,* there are 3 other subspecies namely 1) *Cuora amboinensis amboinensis* (East Indian Box Turtle, Wallacean Box Turtle) in which the distribution is around Sulawesi and northeastern Indonesia and Philippines, 2) *Cuora amboinensis couro* (Indonesian Box Turtle; synonymy as Emys couro [Schweigger, 1812] and Terrapene bicolor [Bell, 1826]) in which the distribution is around Sumatra and southern Indonesia, and 3) *Cuora amboinensis lineata* (Burmese Box Turtle) (McCord and Philippen, 1998) which is distributed around northern Myanmar.

1.2 Morphology of Malayan Box Turtle

They are most easily recognized by their dark olive or black coloured head, with three yellow stripes running along each side (Schoppe, 2011). Besides that, the carapace is dark olive, brown to black, whereas the plastron is yellow, cream, or pale brown with or without dark blotches

on the marginal or on the outer edges of the plastral scutes (Schoppe, 2011). *C. a. kamaroma* has a high-doomed shell and not elongated as other species such as *C. a. amboinensis, C. a. couro* and *C. a. lineata.* They can only be differentiated sexually when they achieve adulthood which is around 4 to 5 years of age. According to Tabaka (2003), male box turtle has larger and thicker tail as compared to female box turtle which has a short, stubby tail. The male also has slight plastral concavity as compared to female as they have flat plastron.

1.3 Status of Malayan Box Turtle in the Wild and in the Market

According to Azrina and Lim (1999), Malayan Box Turtle is considered the most common turtle in the wild and in the markets in Borneo and Peninsular Malaysia, and common and vulnerable in Selangor around 15 years ago. Due to the unsustainable trade in this species, they were listed in 2000 on Appendix II of the Convention on International Trade in Endangered Species (CITES), and thus trade should be strictly controlled and monitored.

This study was undertaken to fulfill the following objectives:

I. to isolate the normal flora from the cloaca of the Malayan Box turtle, and
II. to identify and differentiate the types of the normal flora of the Malayan Box turtle whether they are opportunistic or zoonotic bacterial organisms.

2.0 LITERATURE REVIEW

2.1 Normal Flora of the Gastrointestinal Tract of Malayan Box Turtle

Malayan Box turtle has the same anatomical structure of gastrointestinal tract with other species and type of reptiles. The gastrointestinal tract extends from mouth to cloaca. The length of the gut differs according to the diet consumed by the turtles.

Many bacteria have been identified as causing illness in turtles kept in captivity (Glazebrook *et al*., 1993). However, it is known that many organisms can cause high mortality in other species of free-living marine animals (Gulland, 1999).

Wild turtles are believed to shed *Salmonella* at lower rates than captive turtles because they either lack exposure to stressors that increase shedding rates or because they are not natural carriers of the bacterium (Saelinger, 2006). It has been shown that captivity increases the levels of corticosterone, which also leads to decreased immunocompetence.

According to Snipes *et al*. (1980), *Pasteurella testudinis* have been found to be part of the gastrointestinal and nasal flora of healthy tortoises. Thus, this indicates that it is a commensal inhabitance of the mucosal membrane of the respiratory and alimentary tract with possible opportunistic pathogenic capabilities (Jang and Biberstein, 1991).

2.2 Zoonotic Pathogens of Reptiles

Gram-negative bacteria are commonly reported pathogens in reptiles, whereas disease associated with gram-positive bacteria is sporadic (Rosenthal and Mader, 1996). *Salmonella* spp. can cause reptile-associated salmonellosis in people, particularly in children under 5 years old. This continues to be a growing concern for public health agencies, considering the Centers for

Disease Control and Prevention (CDC) estimates 1.4 million cases annually and reptile-associated salmonellosis accounts for 5% (74,000) of those cases per year (CDC 2002) in United States.

The majority of reptile-associated salmonellosis cases are infections resulting from contact with common pets (Mermin *et al*., 1997). In one of the studies, they have discovered that several of the bacteria found in the cloacal cavity of crocodiles are potential pathogens and could affect water quality and human health. Johnston *et al*. (2010) studied bacteria from the cloaca of the American alligator *Alligator mississippiensis* as well as bacteria from surface water samples from their habitat, and they found similar flora present in the cloaca of alligator and the water they inhabit. They concluded that alligators are a potential source of bacterial contamination of their aquatic habitat through the excretion of feces. Hence, bacteria found in American and Morelet's crocodiles can also be expected to be present in their respective environments, and several of these bacteria are opportunistic pathogens that may cause wound or enteric infections in humans during activities in water or mud (Noonburg, 2005).

3.0 MATERIALS AND METHOD

3.1 Samples selection

A total of 8 adult Malayan box turtles were chosen and samples of cloacal swabs were collected from the turtles. A sterile transport swab (Cary Blair Transport Medium) was used and the cloacal opening was then sterilized by using alcohol swab to avoid any contamination. The swab was then inserted gently into the cloaca and the sample was then collected by swabbing along the cloacal wall. The samples were then placed into sterile transport tube and brought to the laboratory within 12 hours.

3.2 Bacteria Isolation

Samples of cloacal swab were inoculated onto blood agar and Mac Conkey agar. The media were then incubated at for 24 hours. After 24 hours, the colonies that grew onto the media were identified according to their sizes, colour and shape. Each and every colony of different characteristics was then inoculated onto different blood agar for further subculturing of the bacteria. The agars were then incubated at for 24 hours. After 24 hours, the growths of the colony were then inspected and Gram staining was done. Standard procedures were used for bacterial isolate identification, including Gram stain reaction and colony morphology.

3.3 Gram Stain

Gram staining is a technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by colouring these cells red or violet. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram negative bacteria stain red, which

is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the decolouring process.

One single colony was taken from pure culture media by using a sterile inoculating loop onto a glass slide. The sample was heat fixed by carefully passing the slide through a Bunsen burner a few times. After it had dried, crystal violet was flooded onto the sample and left for 1 minute. The slide was then rinsed with a gentle stream of water to remove the crystal violet. After that, Lugol's iodine was flooded onto the sample that was stained by crystal violet and left for 1 minute. The sample was then rinsed by using water and acetone was added onto the slide for 3 seconds. The acetone then was quickly rinsed by using water and carbol fuschin was added onto the slide by flooding it onto the sample for 1 minute. The sample was then washed with a gentle stream of water to remove the satin. If the bacteria are Gram positive, it will retain the primary stain (crystal violet) and not take the secondary stain (carbol fuschin), causing it to look violet/purple under a microscope. If the bacteria are Gram negative, it will lose the primary stain and take the secondary stain, causing it to appear red when viewed under a microscope.

3.4 Gram Positive Test

3.4.1 Catalase Test

After Gram staining, Gram positive colony was then tested for catalase test. The catalase test is primarily used for gram positive bacteria and can for instance be utilized to distinguish *Staphylococcus* spp. and *Micrococcus* spp., which are catalase positive from *Streptococcus* spp. and *Enterococcus* spp., respectively, which are catalase negative. The bacteria were collected from one colony with a sterile inoculating loop and applied on a microscope slide. One drop of 3% of H_2O_2 was then added to the bacteria and the suspension was observed for any gas formation.

Positive test result will show gas formation in which indicating that the bacterium has catalase. Negative result will show no gas formation.

3.4.2 Slide Coagulase Test

Coagulase test is used to differentiate *Staphylococcus aureus* (positive) from Coagulase Negative *Staphylococcus* (CONS). Slide coagulase test is done to detect bound coagulase or clumping factor. Clumping factor directly coverts fibrinogen to fibrin causing agglutination. A colony is emulsified in a drop of distilled water on a clean and grease free glass slide with a minimum of spreading. Suspensions of control positive and negative strains were done to confirm the proper reactivity of the plasma. A drop of undiluted plasma at room temperature is placed into the suspension. Positive result will show as clumping or agglutination within 10 seconds and negative result if there is no clumping or clumping more than 10 seconds.

3.4.3 Methylene Red/Vogeus-Prokeur Test

Vogeus-Prokeur test was performed to determine the ability of the organisms to produce neutral end product acetyl methyl carbinol (acetoin) from glucose fermentation. Inoculate pure culture of the test organism into MR/VP broth and incubated aerobically at 37°C. Following 24 hours of incubation, 2 mL of the broth was placed onto a clean test tube and the remaining broth was re-incubated for an additional 24 hours. Six drops of 5% alpha-naphthol was added and mixed well. Then, 2 drops of 40% potassium hydroxide, and mixed well to aerate. A pink-red color at the surface was observed within 30 minutes and the tube was shaken vigorously

3.4.4 Mannitol Test

A tube of mannitol medium containing glucose (0.5%) was inoculated with bacteria by using wire loop. The inoculum was ensured to reach bottom of the tube. The surface of agar was covered with layer of sterile paraffin oil at least 25 mm thick. The tube was incubated for 5 days at 37°C. Acid is produced anaerobically if indicator changes to yellow throughout tube, indicating presence of *S. aureus*.

3.5 Gram negative test

3.5.1 Oxidase test

Oxidase test was used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. A strip of filter paper is soaked with a little freshly made 1% solution of the reagent. A loopful of culture was rubbed on it with a platinum loop. A positive reaction was indicated by an intense deep-purple hue, appearing within 5-10 seconds, a "delayed positive" reaction by colouration in 10-60 seconds, and a negative reaction by absence of colouration or by colouration later than 60 seconds.

3.5.2 Triple Sugar Iron (TSI) Test

Triple sugar iron (TSI) agar was used for the differentiation of Enterobacteriaceae cultivated on selective or moderately selective media on the basis of lactose, glucose, and sucrose fermentation, and the production of hydrogen sulfide (H_2S) and other gases. A test tube of TSI media was inoculated by stabbing the butt with a needle which had touched the surface of the center of a colony and a slope was then streaked. After 24 hours of incubation, the tube was examined for the following: only acid, or acid and gas in the butt; acid or alkaline or no change in

the slope; and H_2S production. Acidification was detected as yellow-colored gas production by the formation of small bubbles in the agar, and H_2S production by blackening of the medium.

3.5.3 Sulphide, Indole, Motility (SIM) test

Sulphide, Indole, Motility (SIM) test was performed to allow the detection of sulfide production, indole formation and motility of the bacteria. The media will contain ferrous ammonium sulfate and sodium thiosulfate which act as indicator for the production of hydrogen sulfide. Hydrogen sulfide production was detected when ferrous sulfide, a black precipitate, was produced as a result of ferrous ammonium sulfate reacting with H_2S gas. Isolated colonies were taken from solid media such as blood agar is inoculated into the SIM medium by stabbing the center of the medium to a depth of 1/2 inch. The medium then was inoculated at 35°C for 24 hours. After 24 hours, any production of H_2S and motility of the bacteria was then observed. After that, indole test was performed.

Indole tests were also performed which screen for the ability of an organism to degrade the amino acid tryptophan and produce indole. A tube of tryptone broth was inoculated with a small amount of pure culture and incubated at 35°C for 24 hours. To test for indole production, a few drops of Kovac's reagent were added directly to the tube. A positive indole test was indicated by the formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent. If a culture was indole negative, the reagent layer remained yellow or became slightly cloudy.

3.5.3 Citrate Test

Citrate utilization tests were used to identify bacteria, which utilize as one of their starting products of metabolism a compound called citrate, which is the ionized form of citric acid.

Bacterial colonies were picked up with a straight wire and inoculated into a slope of Simmons' citrate agar and incubated for 24 hours at 35°C. As citrate was utilized from the media by the bacteria, the pH of the medium changed. As the pH became more alkaline, the media changed from green to blue (alkaline). A green color represented a negative result, and blue colour shows a positive state of growth.

3.5.4 Urease Test

Urease test was used to determine the ability of an organism to split urea, through the production of the enzyme urease. Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift was detected by the color change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. Rapid urease-positive organisms turn the entire medium pink within 24 hours. The surface of a urea agar slant was streaked with a portion of a well-isolated colony. It then was incubated in 35°C for 24 hours. The development of a pink colour was then examined which shows positive result for urease test.

4.0 RESULTS

4.1 Samples Collection

Samples were collected from the cloaca of 8 adult, healthy Malayan Box turtles. Before samples were collected, the turtles were observed for any signs of sickness or abnormal behaviour. They were kept as indoor pets and fed with fish, vegetables and fruits. The profile of the sampled turtles is shown in Table 4.1

Table 4.1: Data of sampled turtles indicating ID, sex, age and health status.

No.	ID	Sex	Age	Health status
1.	K1	Male	Adult	Healthy
2.	K2	Female	Adult	Healthy
3.	K3	Female	Adult	Healthy
4.	K4	Male	Adult	Healthy
5.	K5	Male	Adult	Healthy
6.	K6	Female	Adult	Healthy
7.	K7	Male	Adult	Healthy
8.	K8	Female	Adult	Healthy

4.2 Bacteria Isolation and Identification

From 8 samples taken, 20 pure colonies were isolated from the blood agar. The colonies were then stained by using Gram staining. From the Gram staining, 17 of the colonies were identified as Gram negative bacteria and 3 of Gram positive bacteria (Table 4.2).

Table 4.2: Gram staining results from each colony of isolates from the samples.

No.	Sample ID	Colony	Gram
1	K1	K1C1	-
2	K1	K1C2	-
3	K1	K1C3	+
4	K2	K2C1	-
5	K2	K2C2	-
6	K2	K2C3	+
7	K3	K3C1	+
8	K3	K3C2	-
9	K3	K3C3	-
10	K4	K4C1	-
11	K4	K4C2	-
12	K5	K5C1	-
13	K5	K5C2	-
14	K5	K5C3	-
15	K6	K6C1	-
16	K6	K6C2	-
17	K7	K7C1	-
18	K7	K7C2	-
19	K8	K8C1	-
20	K8	K8C2	-

For Gram positive bacteria, catalase test was then performed. All 3 bacteria colonies gave positive test results which showed gas formation. This shows that the bacteria are not of *Streptococcus sp.* family. Coagulase slide test was then preceded to all 3 bacteria colonies which all show positive results. All 3 colonies showed agglutination or clumping of the cells by 'lumpy' appearance of the slide. Biochemical test was done to further determine the species of *Staphylococcus sp.* in which all resulted to one species of *Staphylococcus* which is *Staphylococcus aureus* (Table 4.3).

For Gram negative bacteria, oxidase test was then preceded in which 4 colonies showed positive results by changing colour to blue or purple, while the other 13 colonies showed no change in colour upon subjection to oxidase test. Boichemical test which were TSI, SIM, citrate and urea test were done for identification of the bacteria species

From the 8 cloacal samples taken, 9 species of bacteria were able to isolate and identify (Table 4.3). Only one of 9 species was identified as Gram positive bacteria which is *Staphylococcus aureus*, while the rest of 8 species were Gram negative bacteria identified as *Escherichia coli, Acinetobacter lwoffi, Serratia sp., Pantoea agglomerans, Pasteurella testudinis, Klebsiella oxytoca, Acinetobacter calcoaticus* and *Salmonella* spp. The most prevalent bacteria species was *Pantoea agglomerans* (20%), followed by *Staphylococcus aureus* (15%), *Acinetobacter lwoffi* (15%), *Pasteurella testudinis* (10%), *Klebsiella oxytoca* (10%), *Serratia sp.* (10%), *Salmonella spp.* (10%), *E. coli* and *Acinetobacter calcoaticus* (5.0%) (Figure 4.1).

Table 4.3: Bacteria that were able to be isolated from the samples.

No.	Colony ID	Bacteria species
1.	K1C1	*Escherichia coli*
2.	K1C2	*Acinetobacter lwoffi*
3.	K1C3	*Staphylococcus aureus*
4.	K2C1	*Serratia* sp.
5.	K2C2	*Pantoea agglomerans*
6.	K2C3	*Staphylococcus aureus*
7.	K3C1	*Staphylococcus aureus*
8.	K3C2	*Pasteurella testudinis*
9.	K3C3	*Pantoea agglomerans*
10.	K4C1	*Klebsiella oxytoca*
11.	K4C2	*Pantoea agglomerans*
12.	K5C1	*Pasteurella testudinis*
13.	K5C2	*Serratia* sp.
14.	K5C3	*Klebsiella oxytoca*
15.	K6C1	*Acinetobacter lwoffi*
16.	K6C2	*Acinetobacter calcoaticus*
17.	K7C1	*Pantoea agglomerans*
18.	K7C2	*Salmonella* spp.
19.	K8C1	*Acinetobacter lwoffi*
20.	K8C2	*Salmonella* spp.

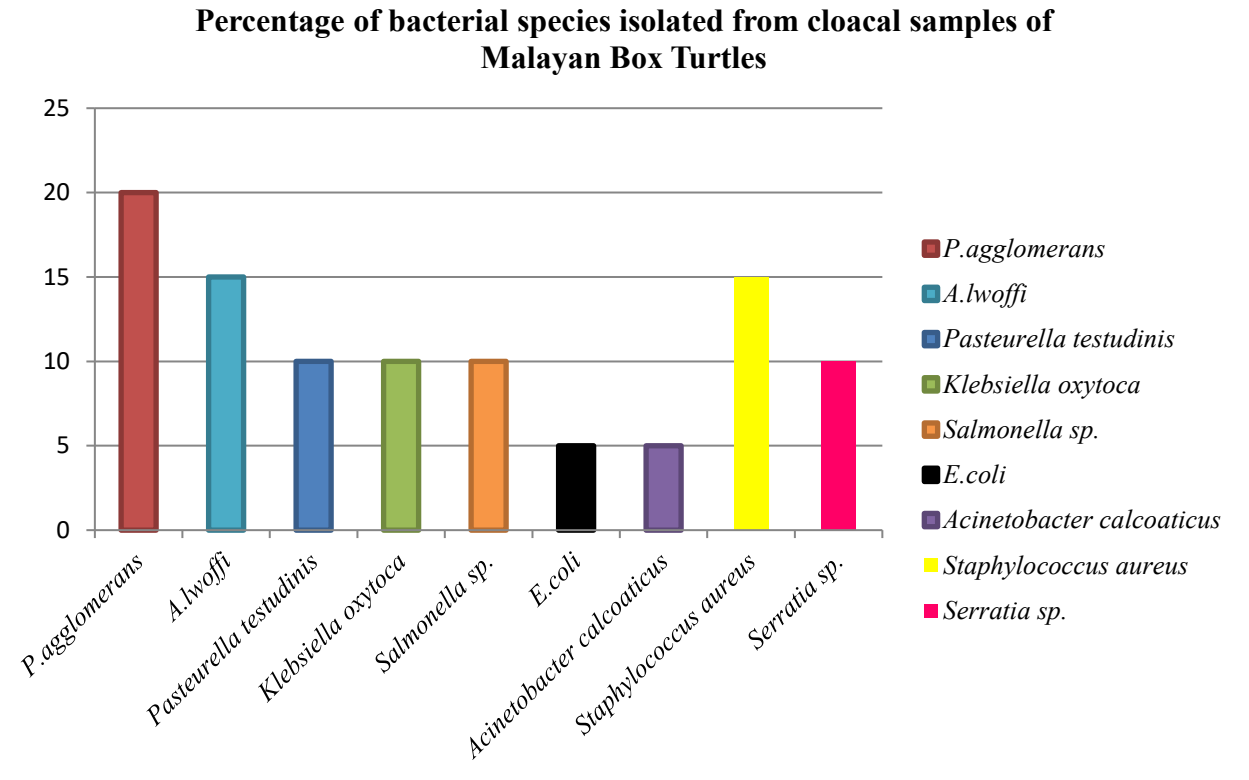

Figure 4.1 Percentage of bacterial species isolated from cloacal samples of Malayan Box turtles

The comparison among bacterial species in male turtles and female turtles was tabulated to identify and differentiate species of bacteria that commonly isolated from each gender (Figure 4.2). From the histogram, it could be observed that *K. oxytoca* and *E. coli* were able to isolate from male turtles but not from the females, while *Acinetobacter calcoaticus* was only able to isolate from the females. The most common bacteria that can be found in the males are *Klebsiella oxytoca, Pantoea agglomerans* and *Pasteurella testudini,* while in females *Acinetobacter lwoffi, Staphylococcus aureus* and *Pantoea agglomerans* are more commonly presence in the cloaca of the Malayan box turtles.

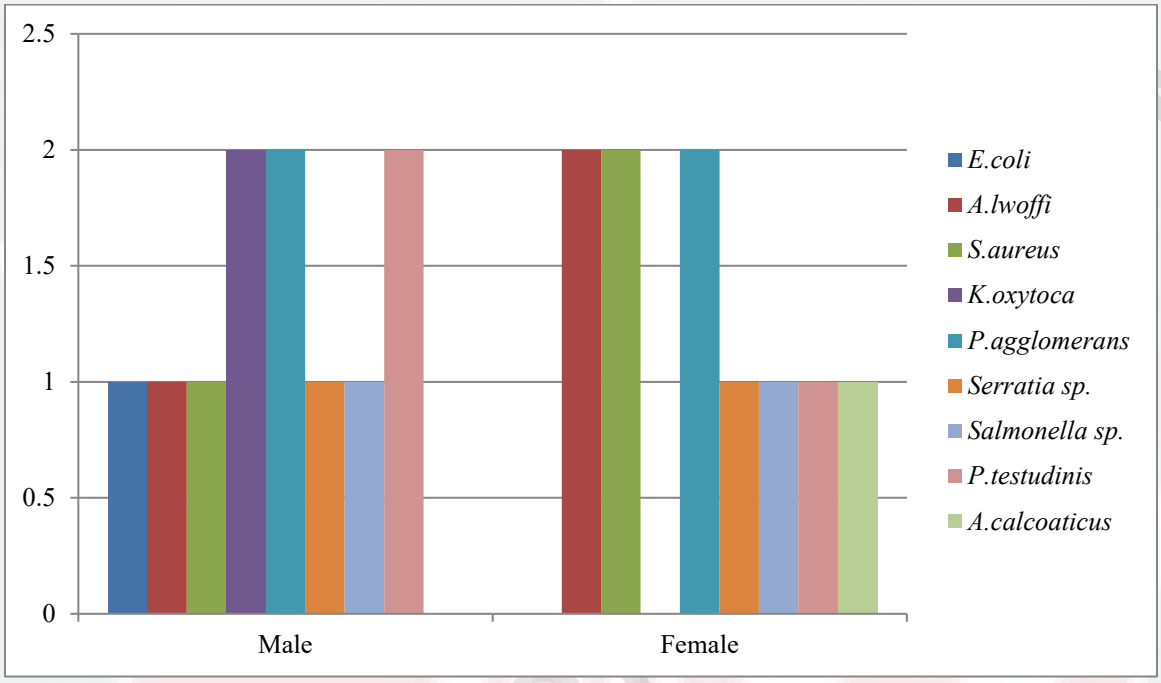

Figure 4.2 Numbers of male and female Malayan Box turtles positive for normal flora and opportunistic bacteria in cloaca

5.0 DISCUSSION

From the results, it could be seen that the normal flora of the cloaca of healthy Malayan Box turtles consists of many types and species of bacteria. Some of the bacteria such as *Salmonella* spp*., Acinetobacter* sp*., Serratia* sp*. and E. coli* are opportunistic and can cause disease in the animal when they are under stress. Some of the isolates are opportunistic pathogens of humans, especially more dangerous to immunosuppressed individuals such as organ transplanted patients. Zoonotic transmission can occur between animals to human and from animals to animals if they are exposed to the pathogens. Individuals involved with these animals or having any contact with the animals will run the risk of being infected by opportunistic pathogen during inspection, transport, or conducting research. The possibility of infection is higher in persons who are sick or immunocompromised.

5.1 Opportunistic Pathogens from GIT of Malayan Box Turtle

From the results, four species of opportunistic bacteria were able to isolate and identify which are *Acinetobacter lwoffi, Serratia* sp*., Pasteurella testudinis, Acinetobacter calcoaticus* and *Salmonella* spp.. These organisms are capable of causing opportunistic infection in malnourished reptiles or kept at suboptimal environmental temperatures (Mader 1998; Harris and Rogers 2001; Mehler and Bennett, 2003).

The possibility of these organisms cause pathological conditions is dependent on circumstantial details and the most important is the state of stress and comorbidities (Jacobson *et al*., 1998; Raidal *et al*., 1998; Dickinson *et al*., 2001). The numbers of microorganisms that can be isolated are more abundant in diseased individuals (Jacobson *et al*., 1991; Dickinson *et al*., 2001). Such a condition is probably verified because immunocompromised patients are unable to

adequately respond to an infection, thus promoting the massive proliferation of bacteria in healthy individuals.

Snipes *et al*. (1980) have isolated *Pasteurella testudinis*, which is one of the Gram negative bacteria that was identified as a normal flora in the gastrointestinal tract and nasal cavity of healthy tortoises. The bacterium is a commensal inhabitant of mucous membranes of the upper respiratory and alimentary tract with possible opportunistic pathogenic capabilities (Jang, 1991). It is also reported to be associated respiratory lesions in captive tortoises with signs of respiratory disease (Snipes & Biberstein, 1982).

5.2 Zoonotic Potentials of Pathogens to Humans

From the results, there are five species of bacteria that may have zoonotic potentials to humans, which include *Staphylococcus aureus* (15%), *Klebsiella oxytoca* (10%), *Serratia sp.* (10%), *Salmonella* spp. (10%) and *E. coli* (5.0%). These makes up 50% of the isolates are zoonotic to humans especially those that have closed contacts with the turtles.

Staphylococcus aureus is known able to cause a diverse array of life-threatening infections, and its capacity to adapt to different environmental conditions (Waldvogel, 2000). Besides that, it is also known as a potentially pathogenic bacterium, which can behave as opportunistic (Lauckner 1985; Glazebrook and Campbell 1990; Glazebrook *et al*., 1993).

According to Marin *et al.* (2013), free-living turtles showed as a risk factor for *Salmonella* infection in humans especially to that have close in contact with those turtles. *Salmonella* species are known to be associated with ectotherms and also occur in their captive environment. It has been well documented that reptiles may spread *Salmonella* intermittently for a long time without showing any clinical signs of disease (Burnham *et al*., 1998; Mader 1998; Mitchell and Shane,

2000). *Salmonella* spp. are mainly transmitted by the feacal-oral route. They are carried asymptomatically in the intestines of infected reptiles and are continuously or intermittently shed in the feaces. *Salmonella* is most commonly transferred between reptiles by contact with contaminated feaces of other reptiles or contaminated food, water or soil. Transmission may also occur in utero, perinatally, or by ingestion of contaminated prey. Transovarian passage has also been reported. In animals, asymptomatic *Salmonella* infections are common. Estimates of the carrier rate among reptiles vary from 36% to more than 80-90%, and several serovars can be found in a single animal. Some authorities consider most or all reptiles to be *Salmonella* carriers. Deaths or disease are occasionally reported in reptiles, but seem to be rare.

According to Johnson-Delaney (2006), *E. coli* is considered to pose a potential health hazard to immunosuppressed and/or immunocompromised humans especially those that have contacted with turtles without any protections. It is more likely to be recovered in reptiles living in associations with humans (Gordon & Cowling, 2003). Those that were kept as pets are more likely to shed *E. coli* especially when they are under stressful condition or immunosuppressed.

6.0 CONCLUSION

In conclusion, there is presence of normal flora from the cloaca of Malayan Box turtles. Some of the bacteria have zoonotic potentials and can cause detrimental effects to humans that have contacted with the animal or the pathogens. Appropriate hygienic precautions need to be taken during handling and transporting of the animal to prevent any transmission of zoonotic bacteria from the animal to human.

7.0 RECOMMENDATIONS

As for the recommendations, a larger sample size is needed to be collected for more data in the future. Besides that, comparing healthy to sick animals can also be done as some of the bacteria are opportunistic, thus they can be found in higher number in sick animals. Comparing between cloacae and choanae samples can help to collect more data as some of the bacteria can only be found from the choane of the turtles.

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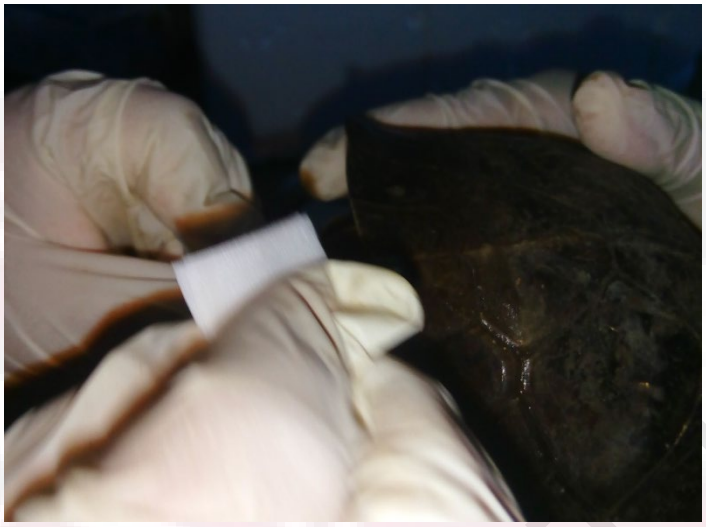
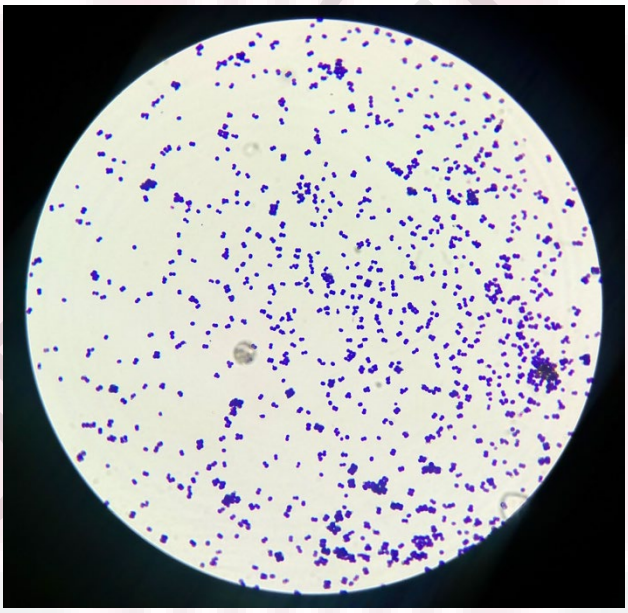
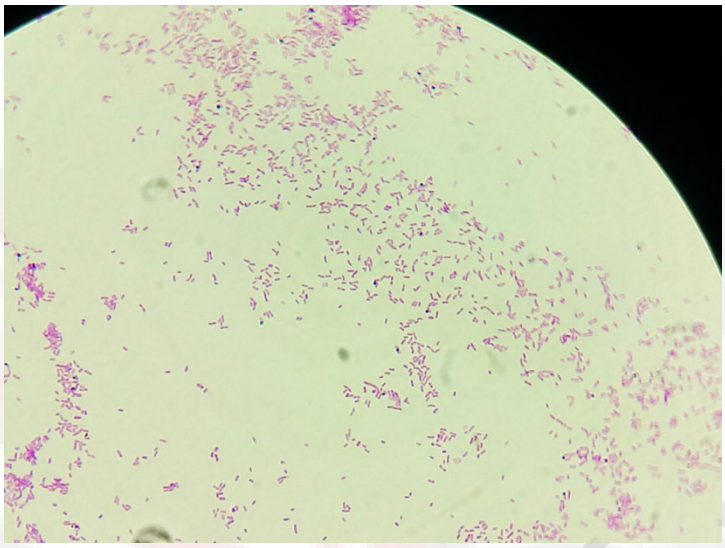
APPENDICES



Appendix 1: Preparation of sample taking by cleaning the opening of the cloaca by using alcohol



Appendix.2: Gram positive bacteria observed under microscope after Gram staining procedure



Appendix 3: Gram negative bacteria observed under microscope after Gram staining procedure

Appendix 4: Biochemical tests for determination of Gram negative bacteria species