UNIVERSITI PUTRA MALAYSIA

ANTIMICROBIAL PROPERTIES OF SERUM FROM CAPTIVE ESTUARINE CROCODILE (*Crocodylus porosus*) AND FALSE GHARIAL (*Tomistoma schlegelii*)

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ANTIMICROBIAL PROPERTIES OF SERUM FROM CAPTIVE ESTUARINE CROCODILE (*Crocodylus porosus*) AND FALSE GHARIAL (*Tomistoma schlegelii*)

ADILAH BINTI ISHAK

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It is hereby certified that we have read this project paper entitled "Antimicrobial Properties of Serum from Captive Estuarine Crocodile (*Crocodylus porosus*) and False Gharial (*Tomistoma schlegelii*)", by Adilah Binti Ishak and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Project.

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ABSTRAK

Abstrak kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Putra Malaysia untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

CIRI ANTIMIKROBIAL DALAM SERUM KURUNGAN BUAYA TEMBAGA (*Crocodylus porosus*) DAN BUAYA JEJULUNG (*Tomistoma schlegelii)*

Oleh

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Mac 2018

Penyelia: Dr Tengku Rinalfi Putra Tengku Azizan

Serum buaya tembaga (*Crocodylus porosus*) dan buaya jejulung (*Tomistoma schlegelii)* diketahui mempunyai ciri antimikrobial. Ini membolehkan buaya hidup di paya dan di kawasan yang boleh menyebabkan penyakit tanpa ancaman yang boleh membawa maut. Satu kajian mengenai serum dari kurungan buaya tembaga dan buaya jejulung telah dijalankan untuk mengkaji ciri antimikrobial. Sampel darah dari

2 buaya tembaga dan 2 buaya jejulung diambil daripada urat ekor dorsal dengan menggunakan jarum 5 inci 18 G 10ml jarum suntik. Darah dibiarkan beku dan emparan. Sampel serum buaya tembaga and buaya jejulung diambil untuk menentukan ciri antimikrobial terhadap 6 bakteria patogen dengan melakukan kaedah peresapan tunggal dan pemerhatian zon perencatan pada Mueller Hinton agar. Zon perencatan diperiksa, diukur, direkodkan dan dianalisis selepas inkubasi 24 jam di 37ºC. Hasilnya, tiada ciri antimikrobial terhadap *Escherichia coli, Salmonella sp, Staphylococcus aureus, Klebsiella pneumoniae, Enterobacter cloacae* dan *Pseudomonas aeruginosa*. Sebagai kesimpulan, tidak ada ciri antimikrobial dalam serum dari kurungan buaya tembaga dan buaya jejulung di dalam kajian ini.

Kata kunci: buaya tembaga, buaya jejulung, ciri antimikrobial

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, UPM in partial requirement of the course VPD 4999 – Project.

ANTIMICROBIAL PROPERTIES OF SERUM FROM CAPTIVE ESTUARINE CROCODILE (*Crocodylus porosus*) AND FALSE GHARIAL (*Tomistoma schlegelii*)

By

Adilah Binti Ishak

Mac 2018

Supervisor: Dr Tengku Rinalfi Putra Tengku Azizan

The serum of Estuarine Crocodile (*Crocodylus porosus*) and False Gharial (*Tomistoma schlegelii*) were known to have antimicrobial properties. This allows crocodiles to live in harsh and highly pathogenic environment without the threat of being fatally infected. A study on serum from captive Estuarine Crocodile and False Gharial was conducted to examine the antimicrobial properties. Blood samples from 2 Estuarine Crocodiles and 2 False Gharials were collected from the dorsal tail vein by using a 5 inch 18 G needle of 10 ml syringe. The blood was left to clot and centrifuged. The serum samples of Estuarine Crocodile and False Gharial were collected to determine the antimicrobial properties against 6 pathogenic bacteria by performing disc diffusion method and observation of inhibitory zone on Mueller Hinton agar. The zone of inhibition was examined, measured, recorded and analysed after 24 hours of agar incubation at 37ºC. The results showed no antimicrobial properties against *Escherichia coli, Salmonella sp, Staphylococcus aureus, Klebsiella pneumoniae, Enterobacter cloacae* and *Pseudomonas aeruginosa*. In conclusion, there are no antimicrobial properties in serum from captive Estuarine Crocodile and False Gharial in this particular study.

Keywords: Estuarine Crocodile, False Gharial, antimicrobial properties

1.0 INTRODUCTION

Archosaurs or the ruling reptiles is a remarkable group that comprised of Crocodilians which also include the defunct thecodonts, fossil of reptiles with four legs or two legs in the Triassic period with teeth set at the sockets's jaw, its earliest and most primordial associates, the pterosaurs or flying reptiles, the dinosaurs, and the ancestors of birds. The class of Reptilia conventionally has a subclass of Archosauria, yet in the meantime the categorization of reptiles is at the state of inconstancy and the taxonomic status of a few major groups may have to be reconsidered (Bellairs A. [d'A.], 1987; Benton, 1982; Green *et. al*., 2014; Oxford Online, n.d.)

Each and every crocodilian at this time belongs to the Eusuchia. The Crocodylidae is a sole family that comprises of some 27 species and subspecies which can be grouped accordingly. This family is categorised into 3 subfamilies which are the Alligatorinae such as Alligator, Caiman, *Melanosuchus*, *Paleosuchus*, Crocodylinae such as genera *Osteolaemus* and *Crocodylus*, and lastly Gavialinae such as *Gavialis and Tomistoma* or false gharial that formerly has been considered as associating to the Crocodylinae (Bellairs A. [d'A.], 1987; Taplin, 1984; Oaks, 2007).

Animals have a prominent distinction in behaviour, morphology, and immune system as to survive and to fit in the environment. Crocodile is a powerful animal that enables it to survive in an extreme environment that harbours pathogenic disease to human or other animals.

Territorial behaviour usually ends up in aggresion that results in flesh wounds. Serious injuries usually involve the loss of the limbs in these disputes. Nevertheless, the injuries tend to recover frequently without infection in spite of the high concentration of potentially pathogenic microorganisms environment (Merchant *et. al.*, 2006).

As reported by Adam Britton, a biologist in North Australia, he has discovered crocodilians in the crocodiles' blood which is almost identical to the antimicrobial proteins. The provision of innate immune system is due to the existence of antimicrobial peptides in alligators and crocodiles that give automatic defense from a particular disease (Amitabh, 2008).

1.1 OBJECTIVE

To determine the antimicrobial properties of serum from captive Estuarine Crocodile (*Crocodylus porosus*) and False Gharial (*Tomistoma schlegelii*).

1.2 HYPOTHESIS

H_0= No antimicrobial properties in serum from captive Estuarine Crocodile (*Crocodylus porosus*) and False Gharial (*Tomistoma schlegelii*).

H_1= There are antimicrobial properties in serum from captive Estuarine Crocodile (*Crocodylus porosus*) and False Gharial (*Tomistoma schlegelii*).

1.3 JUSTIFICATION

The antimicrobial compound in the crocodilians to kill bacteria has a potential in human therapeutic use.

2.0 LITERATURE REVIEW

2.1 Estuarine crocodile (*Crocodylus porosus*)

The saltwater or Indo-pacific crocodile (*Crocodylus porosus*) is the greatest and terrified among of all the crocodilians. It is also known as saltwater crocodile as it is mainly discovered in brackish or saltwater zones. This saltwater crocodile can take up saltwater without detrimental outcome due to the well develop of its salt glands (Rue, 1994). It broadens up to the grass-swamp and freshwater rivers although it is usually related with brackish water (Groombridge, 1987). It was reported of having a length of 6 to 7 metres whereby it is regarded as the greatest of the living crocodilians (Webb & Manolis, 1989). However, Groombridge (1987) reported that this most gigantic crocodilian can reach about 9 metres length.

The Indo-pacific crocodile lives in the northern Australia, the entire Indonesia and neighbouring islands and area that enclose the lower point of India up to Philippines (Rue, 1994). This species is regarded as rare in Peninsular Malaysia (Webb, *et. al.*, 2010). A relatively density of 2.9 NH/km in Rembau Estuary exhibit occurrence of hatchlings that shows a fortunate nesting in the previous season based on a preliminary survey (Nazli & Hashim, 2009). The saltwater crocodile is regularly seen in Kinabatangan river, Sabah and related wetlands (Webb *et. al.*, 2010). A small yet feasible population about 50 individuals was found upon a suvery done at Klias river (Stuebing & Mohd. Sah,1992). However, a constant lower densities all over Sabah was reported (Cox & Gombek, 1985).

It consumes fish, yet similar to Nile crocodile, it can grasp mammals of almost the size of water buffaloes (Rue, 1994). The mature saltwater crocodile consumes bigger vertebrates of increasing portion whilst the younger ones will take up small fish, insects and crustacea.

This species is endangered by the habitat demolition and boundless hunting for skins. The minute size of the belly scales and the comparatively bigger area in the absence of osteoderms lead to a sky-high commercial price of the saltwater crocodile's skin (Groombridge, 1987). Based on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threaten Species, since 1996 the saltwater crocodile is classified as least concerned. It is also listed in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in Appendix I as the most endangered species.

2.2 False Gharial (*Tomistoma schlegelii*)

False gharial (*Tomistoma schlegelii*) has a long, extremely thin snout of the gavial or true gharial and it is a freshwater crocodile. But it has a corpulent body unalike the true gharial. The false gharial is non-dangerous to human and can increase in size around 6 feet or 1.8 metres. The wild population is scarcely discovered in Borneo, Sumatra, and Malay Peninsula whereby it is also an endangered species. The key for the decreasing in the amount of this crocodile is due to loss of its natural territory (Rue, 1994).

This slender-snouted, moderately huge, fish-eating species live in swamps, lakes and freshwater rivers (Groombridge, 1987). Peat swamp and freshwater swamp forest are the most reported records which surround by eastern Sumatra, Peninsular Malaysia and most of the lowlands of Borneo (Stuebing *et. al.*, 2006). It is endangered by habitat alteration and over abusing for skins (Groombridge, 1987).

False gharial is currently listed as vulnerable under the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threaten Species.

2.3 Antimicrobial

The characterization of immune system in crocodilians is not well identified, yet there are a few reports on the capability against bacteria in the serum of American alligator and Siamese crocodile, viral in the serum of American alligator, and fungal in Siamese crocodile blood (Merchant *et. al.*, 2003; Preecharram *et. al.*, 2008; Merchant *et. al.*, 2004; Leelawongtawon *et. al.*, 2010).

2.4 Immune system

Immune system consists of cells, structures, organs and factors that can be taken into account in the defense mechanism of the host against tumors and pathogens (Origgi, 2007).

There are three groups of immune function that acquire by the reptiles such as cell mediated, humoral and innate. Both humoral and cell mediated functions can be categorised under the acquired immunity. This acquired immunity requires the identification of individual pathogens and keeps hold of memory of that exposure. Innate immunity does not involve any identification of unique or individual pathogens. Thus, memory of preceding exposure is not required (Keller *et. al.*, 2016).

A few factors can affect the experimental immunization or natural immunization in the seroconversion of reptiles. These include the conditions of the environment, nature of the pathogen, and biology of the particular reptiles. Some reptiles acquire several months for a maturation of immune response whereas others have a competent immune system at birth (Jacobson & Origgi, 2007).

3.0 MATERIALS AND METHODS

3.1 Animals

The serum of 2 adult estuarine crocodiles were obtained from Sarang Buaya in Pasir Gudang, Johor and 2 adult false gharials from a captive pond at Sungai Dusun owned by PERHILITAN.

3.2 Serum samples

The animals were physically restrained using ropes during blood sampling. Blood was collected via dorsal tail vein using a 5 inch 18 G needle of 10 ml syringe. The collected blood then was transferred into plain tubes and allowed to clot at room temperature. The clotted blood was removed and separated from the serum by centrifuging at 1000 G for 10 minutes. The supernatant was transferred into the polypropylene tube by using Pasteur pipettes. The samples were transported and stored at -20°C prior to the usage for antibacterial properties assay. Procedures involving animals were carried out with the approval by the Institutional Animal Care and Use Committee (IACUC), University Putra Malaysia

3.3 Preparation of cultures and inoculation

A total of 6 pathogenic bacteria were selected and used in this research. The bacteria include *Escherichia coli, Salmonella sp, Staphylococcus aureus, Klebsiella pneumoniae, Enterobacter cloacae* and *Pseudomonas aeruginosa.* The pure cultures were obtained from stock cultures at Bacteriology Laboratory. The cultures were reconfirmed by culturing and using series of biochemical tests. The bacteria were subcultured on tryptone soy agar and incubated in 37°C for a night.

3.4 Antibacterial properties assay

All cultures was standardised to 0.5 Mc Farland. This was done by inoculating 3 colonies of bacteria into the sterile water and the solution was vortexed until the turbidity is similar as Mc Farland of 1.5 x 10^8. The standardised bacteria suspension were swabbed all over on 3 different planes onto Mueller Hinton agar by using a sterile cotton swab agar and left to dry. A total of 4 serum samples were tested. The serum samples comprised of estuarine crocodile (*Crocodylus porosus*) and false gharial (*Tomistoma schlegelii*) serum which are archived serums from the anatomy laboratory, Faculty of Veterinary Medicine. 15 µL of each serum was inoculated onto a sterile plain disc by using a micropipette and left to dry for 2 hours at room temperature. 2 discs of different serum samples were placed on the surface of the Mueller Hinton agar together with a negative control that consists of a sterile water discs and positive controls of various antibiotic discs. The tests were conducted in duplicates. The zone of inhibition was then examined, measured, recorded and analysed after 24 hours of agar incubation at 37^{o}C.

4.0 RESULTS

4.1 Serum antibacterial properties

Table 1 illustrates the zone of inhibition shows by different serum of estuarine crocodile and false gharial respectively against various bacteria species.

Table 1: Zone of inhibition shows by different serum of estuarine crocodile and false gharial respectively against various bacteria species.

Bacterial Species	Zone of Inhibition				Percentage (%)
	Estuarine Crocodile		False Gharial		
	Adult (E1)	Adult (E2)	Adult (F1)	Adult (F2)	
Escherichia coli	No	No	No	No	0
Salmonella sp	No	No	No	No	0
Staphylococcus aureus	No	No	No	No	0
Klebsiella pneumoniae	No	No	No	No	0
Enterobacter cloacae	No	No	No	No	0
Pseudomonas aeruginosa	No	No	No	No	0

The antibacterial properties against different pathogenic bacteria were shown in Table 1. Both serum from saltwater crocodile and false gharial gave the same results.

The serum of saltwater crocodile tested against *Escherichia coli, Salmonella sp, Staphylococcus aureus, Klebsiella pneumoniae, Enterobacter cloacae* and *Pseudomonas aeruginosa* did not show any zone of inhibition. Also, the serum of false gharial tested against *Escherichia coli, Salmonella sp, Staphylococcus aureus, Klebsiella pneumoniae, Enterobacter cloacae* and *Pseudomonas aeruginosa* did not show any inhibitory effects. Amikacin, Imipenem, Gentamycin and Ceftriaxone were used as positive controls and showed inhibition growth against *Escherichia coli, Salmonella sp, Staphylococcus aureus, Klebsiella pneumoniae, Enterobacter cloacae* and *Pseudomonas aeruginosa*.

5.0 DISCUSSION

The data of serum from saltwater crocodile and false gharial respectively do not exhibit antibacterial activity against the tested pathogenic bacteria. In spite of that, the positive and negative controls were justifiable. Hence, the inhibitory effects are validated. Statistics and data on innate immunity of crodilians, primarily estuarine crocodile (*Crocodylus porous*) *and* False Gharial (*Tomistoma schlegelii*) are fewer. Despite the negative results in this particular study, serums from crocodillians were known to have antimicrobial properties. Nevertheless, there were reports on the antimicrobial activity of estuarine crocodile (*Crocodylus porous*), freshwater crocodile (*Crocodylus johnstoni*), and American alligator (*Alligator mississippiensis*) (Merchant & Britton, 2006; Merchant et al., 2005).

In contrast to other crocodilians, both estuarine crocodile and false gharial against *Escherichia coli, Salmonella sp, Staphylococcus aureus, Klebsiella pneumoniae, Enterobacter cloacae* and *Pseudomonas aeruginosa* in this research did not show any inhibitory effect might be associated with a few factors. As for the host factor, while body temperature of the reptile is within its optimal zone, the immune response shows respond maximally. Response is reduced when the temperature is maintained above and below (Cone & Marchalonis, 1972; Tait 1969).

Those reptiles especially living in temperate areas will face seasonal cycles of lymphoid follicular restoration and involution. The cycles may correspond much better towards mating periods compared to changes in surrounding temperature. The sex steroids and corticosteroids levels respectively may be high and antibody levels may be low during these periods (Leceta & Zapata, 1986; Saad & El Deeb, 1990; Zapata *et. al*., 1992).

In human clinical pathology, the serum storage has been identified as a significant factor (Boyanton & Blick, 2002). A same research has been done in the veterinary literature, but restricted to avian and canine serum samples whereby various species exhibit distinction in storage stability (Hawkins et al., 2006; Thoresan et al. 1992). Protein stability has always been complicated.The serum samples of estuarine crocodile and false gharial conducted in this research were archived serum for a few years. The serum samples were refrigerated and stored at -20^{o}C which may lead to protein denatured. A few handlers have been using the serum sample along the period of time for other purpose of research. This can lead to a few freeze-thaw cycle situations of the serum samples. In comparison to human serum, according to Cao et al. (2003), a critical destruction of proteins can arise if slow thawing with fast freezing happen; as a consequence, fast thawing and slow freezing are advocate to avoid denatured of protein in aqueous solution. Also, Mitchell *et. al*., (2005) discovered that long period of storage at -70^{o}C is less complicated compared to repeat thawing of the sample. Hence, the denatured protein of the serum samples of estuarine crocodile and false gharial may cause the possibility of no antibacterial zone of inhibition in this research.

Upon performing antimicrobial susceptibility testing, a disc diffusion method was conducted to observe the inhibitory effect. There are a few factors that may lead to negative results of antimicrobial sensitivity test which include application of disc time, incubation temperature, agar medium pH, unsuitable storage conditions, moist surface of the medium, and density of inoculum (Lalitha, 2014). Besides, this method also has its very own advantages such as Simplicity in test, most economic flexibility in disc selection, and the result can be easily interpreted (Bagul &Sivakumar, 2016).

6.0 CONCLUSION

There are no occurrence of antimicrobial properties from the serum in captive Estuarine Crocodile (*Crocodylus porosus*) and False Gharial (*Tomistoma schlegelii*).

7.0 RECOMMENDATIONS

For further studies, it is recommended to increase the number of sample sizes to obtain a more reliable data. Besides, samples collection from animals in different ponds or captivities should be done to avoid occurrence of bias in order to represent the whole population. Also, a precautious serum storage and handling should be done to avoid protein denaturation. Hence, fresh serum sample is advised to be collected.

REFERENCES

Amitabh A. (2008). Alligator blood may lead to powerful new antibiotics. Retrieved February 5, 2018, from https://news.nationalgeographic.com/news/2008/04/080407-alligator-blood.html

Bagul, U.S., & Sivakumar, S. M. (2016). Antibiotic susceptibility testing: a review on current practices. International Jornal of Pharmacy, 6(3): 11-17

Bellairs, A. [d'A.]. (1987). The crocodilia. In G. Webb, S. C. Manolis & P. J. Whitehead (Eds.), Wildlife management : crocodiles and alligators (pp. 5-7). Published by S. Beatty & Sons in association with the Conservation Commission of the Northern Territory.

Benton, M. J. (1982). The Diapsida: revolution in reptile relationships. Nature, 296: 306-307

Boyanton BL Jr, & Blick KE. 2002. Stability studies of 24analytes in human plasma and serum. Clinal Chemistry 48:2242–2247

Burnie, D. (2011). Animal: the definitive visual guide to the world (pp. 432). Dorling Kindersley.

Cao E., Chen Y., Cui Z., & Foster PR. (2003). Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnology and Bioengineerirng, 82:685-90. http://dx.doi.org/10.1002/bit.10612

Cone R. E., & Marchalonis J.J. (1972). Cellular and humoral aspects of the influence of environmental temperature on the immune response of poikilothermic vertebrates. J Immunol, 108:952-957

Cox, J. & Gombek, F. (1985). A preliminary survey of the crocodile resource in Sarawak, East Malaysia. IUCN/ WWF Project No. MAL 74/85. WWF: Malaysia.

Dzik, JM. The ancestry and cumulative evolution of immune reactions. Acta Biochimica Polonica 2010;57:443–66

Green, R. E., Braun, E. L., Armstrong, J., Earl, D., Nguyen, N., Hickey, G., … Ray, D. A. (2014). Three crocodilian genomes reveal ancestral patterns of evolution among archosaurs. *Science*, *346*(6215). https://doi.org/10.1126/science.1254449

Groombridge, B. (1987). The distribution and status of world crocodilians. In G. Webb, S. C. Manolis & P. J. Whitehead (Eds.), Wildlife management : crocodiles and alligators (pp. 19-21). S. Beatty & Sons in association with the Conservation Commission of the Northern Territory.

Jacobson, E. R., Origgi F.C. (2007). Serodiagnostics. In E.R. Jacobson (Eds.), Infectious diseases and pathology of reptiles: color atlas and text (pp. 392). Florida, FL: CRC Press.

Hawkins M. G., Kass, P.H., Zink,l J.G., & Tell, L.A. (2006). Comparison of biochemical values in serum and plasma, fresh and frozen plasma, and hemolyzed samples from orange-winged amazon parrots (Amazona amazonica). Vet Clinical Pathology, 35:219–225

Keller, J. M., Peden-Adams, M. M., & Aguire, A.A. (2016). Immunotoxicology and implications for reptilian health. In S. C. M. Gardner& E. Oberdorster (Eds.), Toxicology of Reptiles (pp. 201). CRC Press.

Lalitha, M.K. (2004). Manual on antimicrobial susceptibility testing: Indian Association of Medical Microbiologists, 3-4

Leceta, J., & Zapata, A. (1986). Seasonal variation in the immune response of the tortoise Mauremys caspica. Immunol 57:483-487

Leelawongtawon, R., Siruntawineti, J., Chaeychomsri, W., & Sattaponpan, C. (2010). Antibacterial and antifungal activities from Siamese crocodile blood.

Mitchell, B. L., Yasui, Y., Li, C. I., Fitzpatrick, A. L., & Lampe, P. D. (2005). Impact of freeze-thaw cycles and storage time on plasma samples used in mass spectrometry based biomarker discovery projects. Cancer Informatics, 1, 98–104. https://doi.org/10.4137/cin.s0

Merchant, M. E., Roche, C., Elsey, R. M., & Prudhomme, J. (2003). Antibacterial properties of serum from the American alligator (Alligator mississippiensis). Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 136(3), 505–513. https://doi.org/10.1016/S1096-4959(03)00256-2

Oaks, J. R. (2007). Phylogenetic systematics, biogeography, and evolutioanary ecology of the true crocodiles (Eusachia: Crocodylidae: *Crocodylus*).

Origgi, F. C. (2007). Reptile Immunology. In E.R. Jacobson (Eds.), Infectious diseases and pathology of reptiles: color atlas and text (pp. 131). CRC Press.

Preecharram, S., Daduang, S., Bunyatratchata, W., Araki, T., & Thammasirirak, S.

(2008). Antibacterial activity from Siamese crocodile (Crocodylus siamensis) serum. African Journal of Biotechnology, 7(17), 3121–3128. https://doi.org/10.5897/AJB08.316

Rue, L. L. (1994). Alligators & crocodiles: a potrait of the animal world (pp. 67-77). New York, NY: Todtri.

Saad A. H., & El Deeb S. (1990). Immunological changes during pregnancy in viviparous lizard, Chalcides ocellatus. Vet Immunol Immunopathol 25:279-286

Stuebing, R.B., &Mohd. Sah, S.A. (1992). Distribution, population structure and some aspects of the ecology of the estuarine crocodile (*Crocodylus porosus* Schneider) in the Klias River, Sabah. Pp. 149-162 in Crocodiles. Proceedings of the 11th Working Meeting of the IUCNSSC Crocodile Specialist Group. IUCN: Gland.

Stuebing, R. B., Bezuijen, M.R., Auliya, M., & Voris, H.K. (2006). The current and historic distribution of *Tomistoma schlegelii* (the False Gharial) (Müller 1838) (Crocodylia, Reptilia). The Raffl es Bulletin of Zoology 54: 181-197

Tait, N.N. (1969). The effect of temperature on the immune response in cold-blooded vertebrates. Physiol Zool 42:29-35

Taplin, L. (1984). Evolution and zoogeography of crocodilians: a new look at an ancient order. In M. Archer & G. Clayton (Eds.), Vertebrate Evolution and Zoogeography in Australia (pp. 361-370). Perth: Hesperian Press.

Thecodont (n.d.). *Oxford Online.* In Oxford. Retrieved March 7, 2018, from https://en.oxforddictionaries.com/definition/thecodont.

Thoresen, S. I., Havre, G. N., Morberg, H., Mowinckel, P. (1992). Effects of storage time on chemistry results from canine whole blood, heparinized whole blood, serum, and heparinized plasma. Vet Clialal Pathoogyl, 21:88–94

Webb, G & Manolis, C. (1989). Crocodiles of Australia. Sydney: Reed Books.

Webb, G.J. W., Manolis, S. C., & Brien, M.L. (2010). Saltwater crocodile Crocodylus porosus. Crocodiles. Status survey and conservation action plan. Third edition IUCN Crocodile Specialist Group, Darwiin, Australia, 99-113

Zapata A. G., Varas, A., & Torroba M. (1992). Seasonal variation in the immune system of lower vertebrates. Immunol Today 13:142-147

APPENDIX 1

Figure 1: Example of Estuarine Crocodile serum with no zone of inhibition against bacteria.

APPENDIX 2

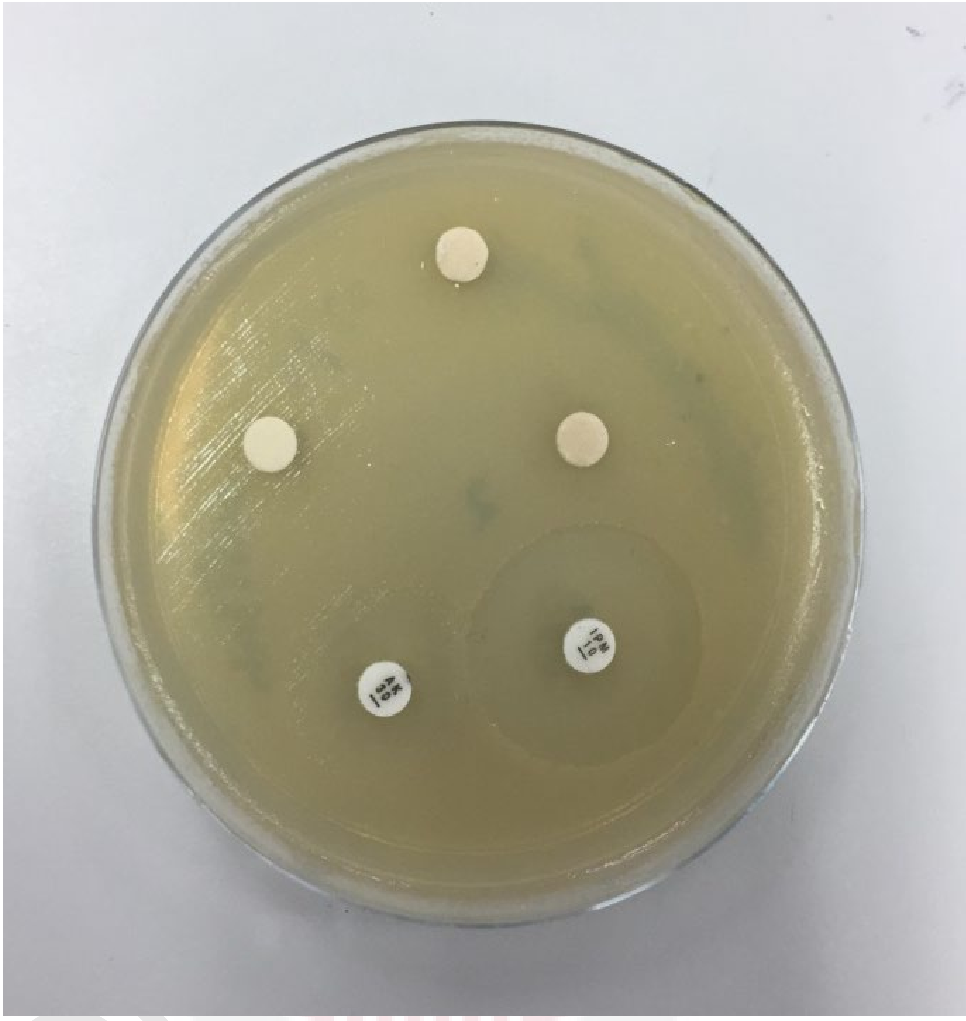

Figure 2: Example of False Gharial serum with no zone of inhibition against bacteria.