DETECTION OF SARS-CoV-2 (COVID-19) IN BAT SAMPLES IN EAST COAST MALAYSIA BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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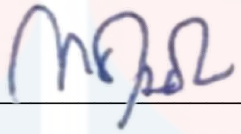
CERTIFICATION

This is to certify that we have read this research paper entitled **'Detection of SARS-CoV-2 (Covid-19) in East Coast Malaysia by Enzyme-Linked Immunosorbent Assay (ELISA)** by Nur Diyana Binti Mohd Rusuki, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course DVT 55204 – Research Project.



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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, University Malaysia Kelantan, in partial requirement on the course DVT 55204 – Research Project.

DETECTION OF SARS-CoV-2 (COVID-19) IN BAT SAMPLES IN EAST COAST MALAYSIA BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

By

Nur Diyana Binti Mohd Rusuki

2022

Supervisor: Assoc. Prof. Dr. Maizan Binti Mohamed

Co-supervisor: Dr. Choong Siew Shean

SARS-CoV-2 is a coronavirus associated with severe acute respiratory syndrome that caused a global COVID-19 pandemic in late 2019. SARS-CoV-2 has been found in bats from Southern Thailand, Japan, China, and Indonesia, according to several investigations. However, no research on surveillance of coronavirus in bats in East Coast Malaysia has been conducted. Hence, this study aims to identify the presence of SARS-CoV-2 in bat samples from East Coast Malaysia through an ELISA test kit. Oropharyngeal swabs in bats from Terengganu, Pahang, and Kelantan were taken as samples and were detected using an ELISA test kit; COVID-19 nucleoprotein ELISA Kit from Wuhan Fine Biotech Co., Ltd. As a consequence, 23 of the 90 samples tested

positive, indicating that SARS-CoV-2 is present in bat samples from Malaysia's East Coast.

Keywords: *Bats, East coast Malaysia, COVID-19, ELISA, SARS-CoV-2*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan.

PENGESANAN SARS-CoV-2 (COVID-19) DALAM SAMPEL KELAWAR DI PANTAI TIMUR MALAYSIA OLEH UJIAN IMUNOSORBEN BERKAITAN ENZYME (ELISA)

Oleh

Nur Diyana Binti Mohd Rusuki

2022

Penyelia: Prof. Madya Dr Maizan Binti Mohamed

Penyelia bersama: Dr Choong Siew Shean

SARS-CoV-2 ialah coronavirus yang dikaitkan dengan sindrom pernafasan akut teruk yang menyebabkan pandemik COVID-19 global pada akhir 2019. SARS-CoV-2 telah ditemui dalam kelawar dari Selatan Thailand, Jepun, China dan Indonesia, menurut beberapa penyiasatan. Bagaimanapun, tiada kajian mengenai pengawasan koronavirus dalam kelawar di Pantai Timur Malaysia telah dijalankan. Justeru, kajian ini bertujuan untuk mengenal pasti kehadiran SARS-CoV-2 dalam sampel kelawar dari Pantai Timur Malaysia melalui kit ujian ELISA. Sapuan oropharyngeal dalam kelawar dari Terengganu, Pahang, dan Kelantan telah diambil sebagai sampel dan dikesan menggunakan kit ujian ELISA; Kit ELISA nukleoprotein COVID-19 daripada Wuhan

Fine Biotech Co., Ltd. Akibatnya, 23 daripada 90 sampel diuji positif, menunjukkan bahawa SARS-CoV-2 terdapat dalam sampel kelawar dari Pantai Timur Malaysia.

***Kata kunci**: Kelawar, Pantai Timur Malaysia, COVID-19, ELISA, SARS-CoV-2*

1.0 Introduction

The Nidovirales order has several virus families, including the Coronaviridae, Arteriviridae, Mesoniviridae, and Roniviridae families. Coronaviruses (CoVs) are the most prevalent of these families. The Coronavirinae and Torovirinae are the two subfamilies that make up the Coronaviridae family. Alpha, beta, gamma, and delta coronaviruses make up the four genera that make up the Coronavirinae family. (Fehr & Perlman, 2015). A coronavirus called SARS-CoV-2 connected to the severe acute respiratory syndrome led to the COVID-19 pandemic in late 2019. In Wuhan, a pneumonia outbreak with an unidentified source was found. The World Health Organization (WHO) identified the epidemic in January 2020, and a pandemic is anticipated in March 2020. (Yang et al., 2020). As for the clinical manifestations, these coronaviruses produce severe respiratory, enteric, neurological, or hepatic disease in their hosts, while human coronaviruses induce respiratory and gastrointestinal problems (Poon et al., 2004).

Bats are mammals belonging to the Chiroptera order and are the only animals capable of genuine and sustained flight. Bats are the second biggest mammalian order (after rodents), with over 1,240 species worldwide (*Bats*, 2017). Bats are the mammals that have the most coronavirus species. In 2019, bats from Africa, Asia, the Americas, and Europe were found to carry more than 200 coronaviruses. According to a recent study, bats may carry at least 3204 coronaviruses, with many more yet to be discovered (Hernández-Aguilar et al., 2022).

Bats are reservoir for SARS-CoV and SARS-CoV-2 viruses as they are genetically similar. As a result, due to the importance of bats in the viral ecology and evolution, several studies have been performed to investigate novel bat-SARS-like CoVs as well as to better understand how these viruses transmit from animal hosts to people (Balboni et al., 2012). Therefore, this project was conducted to determine the presence of SARS-like CoVs that might have public health importance in the future.

2.0 Research problem statement

Since 2019, more than 200 coronaviruses had been discovered in bats spanning Africa, Asia, the Americas, and Europe (Hernández-Aguilar et al., 2022). SARS-CoVs like viruses were identified in bats from Chachoengsao, South Thailand, (Wacharapluesadee et al., 2022) which is quite close to Kelantan, Malaysia. However, to date, no research has been conducted to detect the surveillance of coronavirus in bats in East Coast Malaysia.

3.0 Research questions

3.1 Are SARS-CoV-2 also present in bats in East Coast Malaysia?

3.1 Can ELISA method sensitive to identify the presence of SARS-CoV-2?

4.0 Research hypothesis

4.1 SARS-CoV-2 also present in bats from East Coast Malaysia.

4.2 The presence of SARS-CoV-2 in bats can be identified using the ELISA approach.

5.0 Objectives

5.1 To identify the presence of SARS-CoV-2 in bat samples from East Coast Malaysia.

5.2 To determine the presence of SARS-CoV-2 in bats from East Coast Malaysia using antigen detection ELISA test kit.

6.0 Literature review

6.1 Characteristics of SARS-CoV-2

Coronaviruses are single-stranded RNA viruses with a massive envelope which is the largest genome of any RNA virus, with genome sizes ranging from 27 to 32 kb. The nucleocapsid protein (N) wraps the DNA in a helical capsid, which is subsequently encased in an envelope that consist of: The membrane protein and the envelope protein are functioning in the process of assembling the virus, and the viral entrance into host cells is facilitated by the spike protein (S). Furthermore, coronaviruses can encode an envelope-associated hemagglutinin-esterase protein (HE) (Li, 2016). Coronavirus particles are spherical in shape, having a diameter of 80 to 160 mm (Yang et al., 2020)

The structural spike protein protrudes from the virus surface in massive protrusions which coronaviruses their crown-like look (hence their Latin-derived name ‘corona’ which translate to crown). As for its role, it does not only enable virus entrance; the spike is a crucial factor of viral host range and tissue tropism, as well as a notable trigger of host immune responses. The coronavirus spike comprises a wide ectodomain, a single-pass transmembrane anchor, and a small intracellular tail. (Li, 2016).

They are members of the Nidovirales order and the Coronaviridae family, which is further divided into the *Letovirinae* and *Orthocoronavirinae* subfamilies. The latter contains the genera *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*, which contain

coronaviruses linked to MERS-CoV, SARS-CoV, and SARS-CoV-2. The first two are found in mammals such as bats, while the latter two are mostly found in wild and domestic birds (Li et al., 2020).

6.2 Diagnosis of SARS-CoV-2.

The new real-time PCR assay is a specific, reliable, and sensitive instrument that has the potential to be used for quick screening in bat populations. If traditional qualitative RT-PCR is used as it allows for the sequencing of the obtained products and the genomic characterization of the viruses, a combined approach with quantitative real-time PCR provides valuable information about the epidemiological situation of coronavirus infection in bats, allowing for a more realistic picture of viral prevalence in the population. (Balboni, et al., 2012).

Apart from that, the gold standard for COVID-19 diagnosis is real-time reverse transcriptase-polymerase chain reaction (RT-PCR)-based assays developed to identify SARS-CoV-2 in nasopharyngeal swabs (Chansaenroj et al., 2021). Meanwhile, the specificity of ELISA is 97.9%, which is comparable to earlier tests of the same type (Tozetto-Mendoza et al., 2021).

6.3 Prevalence of Coronavirus in bats.

Bats were recognized as hosts for lyssaviruses and may be potential reservoirs for new human infections such as the Ebola, Marburg, Nipah, and Hendra viruses, and SARS-CoV. At least one of the two semi-nested RT-PCR assays was positive in 41 (19%) of the 221 bat fecal swabs analyzed. The distribution of CoV detections by bat species and location reveals a number of characteristics of coronaviruses in bats. As seen in *Chaerophon spp*., *Miniopterus inflatus*, and *Rousettus aegyptiacus*, a given bat species can harbor several distinct CoVs in the same location; CoVs can also be seen in the same type of bat in different locations (Tong et al., 2022).

SARS-CoV-2 related with coronaviruses in bats in Southeast Asia, according to both molecular and serological evidence supplied by the researchers. CoVs had high genetic similarity to SARS-CoV-2 are widely found in bats in numerous countries and areas in Asia, according to the researchers. SC2r-CoVs have been found in bats from several regions spanning Asia including Iwata Prefecture in Japan, Zhejiang and Yunnan in China, and Chachoengsao, Thailand. Rhinolophus bats have a wide range of distribution, from Europe to Australia, and Thailand has recorded at least 22 Rhinolophus species. (Wacharapluesadee et al., 2022).

7.0 Materials and methods

7.1 Sample collection

As for this FYP project, the sample were obtained from the virology laboratory University Malaysia Kelantan. The study has been approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, University Malaysia Kelantan (UMK/FPV/ACUE/PG/6/2021). There were 90 oropharyngeal swab samples collected from Sekayu (Terengganu), Gunung Reng (Kelantan) and Merapoh (Pahang).

7.2 Enzyme-linked immunosorbent assay (ELISA) test kit.

COVID-19 nucleoprotein ELISA Kit by Wuhan Fine Biotech Co., Ltd is used to identify SARS-CoV-2. Sandwich enzyme-linked immune-sorbent assay technology served as the foundation for this kit. Biological fluids which is oropharyngeal swab sample were used to determine the presence of SARS-CoV-2 antigen.

7.3 ELISA Protocol

7.3.1 Preparation of reagent

The reagents and samples were allowed to come to room temperature for 20 minutes before being used. All samples were duplicated to ensure the accuracy of the data. (Appendix A).

A 30ml concentrated wash buffer was mixed with 750ml of distilled water to make the wash buffer.

One Standard tube (designated as the zero tube) received 1ml of Sample Dilution Buffer before being kept at room temperature for 10 minutes to create the standard. Next, seven Eppendorf tubes were labeled with 1/2 followed by five tubes of its two-fold dilutions (1/4, 1/8, 1/16/ 1/32, 1/64), and blank. Each tube received 0.3ml of the Sample Dilution Buffer. In the first tube, 0.3ml of the Standard solution from the zero tube was introduced and thoroughly mixed. The 0.3ml from the first tube was then transferred to the second tube and properly mixed. 0.3ml from the second tube was transferred to the third tube and was mixed thoroughly, and so on (Appendix B). For the blank control, Sample Dilution Buffer was used.

The Biotin-labeled Antibody working solution was created an hour before the process. The formula 0.1ml/well x the number of wells was used to determine the total volume of working solution needed. Antibody Dilution Buffer was used to dilution the biotin-detection antibody to 1:100 before being thoroughly blended.

The HRP-Streptavidin Conjugate (SABC) Working Solution was prepared thirty minutes before the experiment. The formula 0.1ml/well x the number of wells was used to determine the total volume of working solution needed. The SABC was then combined with SABC Dilution Buffer and diluted to 1:100.

7.3.2 Assay procedure

Sample pooling was conducted for this experiment, combining two samples into one well. This method was done due to the inadequacy of the ELISA test kit and the high cost of the test kit. Only two ELISA test kit was used for this project.

ELISA reaction was conducted according to the manufacturer's procedure. In standard wells, 100 µl of zero tube, first tube, second tube, third tube, fourth tube, fifth tube, sixth tube, and Sample Dilution Buffer (blank) were divided (A1, B1, C1, D1, E1, F1, G1, H1). In the test sample wells, 100µl of diluted sample was added. After that, the plate was covered and incubated at 37°C for 90 minutes. After that, the wells were emptied and cleansed twice with Wash Buffer. Between washes, the wells were not permitted to dry.

In the standards, test sample, and blank wells, 100µl Biotin-labeled antibody working solution was added. The solution was poured into each well from the bottom up by to avoid contact with the sidewall. After that, the plate was covered and incubated at 37°C for 60 minutes. The wells were drained and washed thrice with Wash Buffer, allowing each time for 1-2 minutes in the well.

Next, each well received 100µl of SABC Working Solution conjugate. After that, the plate was sealed and incubated at 37°C for 30 minutes. The wells were drained and washed five times with Wash Buffer, allowing 1-2 minutes each in the well.

Lastly, each well received 90µl of TMB Substrate. After that, the plate was covered and incubated for 10-20 minutes at 37°C in the dark. Aluminium foil was utilised to cover the place to a crutilized dark environment. Then, each well received 50µl of Stop Solution. The colour will immediately turn to yellow (Appendix C). At 450nm, the O.D. was read and recorded.

7.3.3 Data interpretation

The standard curve was shown as the relative O.D.450 of standard solution (Y) against their respective concentration (X) based on the reading of O.D absorbance at 450 nm in the microplate reader (X). Then, as illustrated in Chart 1, the target concentration of the samples can be extrapolated from the standard curve. The sample that shows the concentration above the value of blank (OD 0.217), will be considered as positive result.

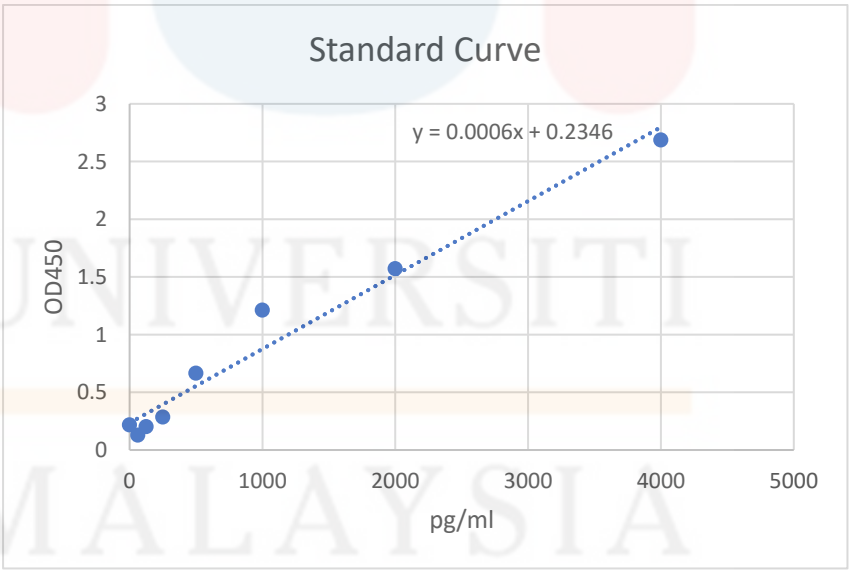


Chart 1: Standard Curve

8.0 Results

According to Table 1, the value of the relative O.D.450 of each standard solution with regard to the standard solution's concentration was noted.

STD (pg/ml)	OD
0	0.217
62.5	0.128
125	0.202
250	0.285
500	0.664
1000	1.211
2000	1.570
4000	2.686

Table 1: O.D.450 of standard solution and respective concentration of the standard solution.

The standard curve was plotted as the relative O.D.450 of standard solution (Y) against their respective concentration (X) as shown in Chart 1.

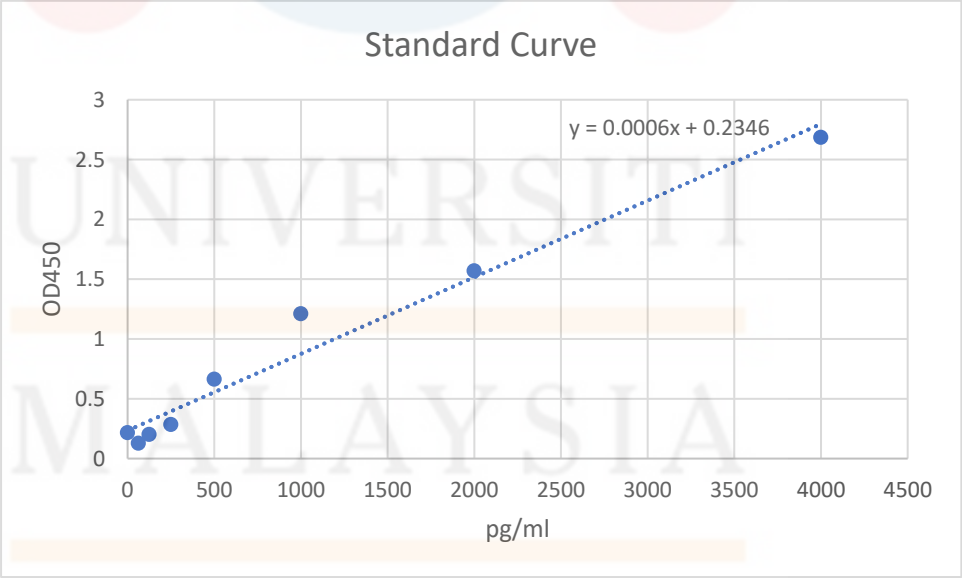


Chart 1: Standard Curve

The concentration of each sample was calculated by using the formula of y=0.0006x+0.2346 that shown in standard curve (Chart 1) and the data were summarized and recorded in Table 2.

Total sample	**Result**	
	Positive	**Negative**
90	23	67

Table 2: Summary of positive and negative result of ELISA

Based on the calculation of each sample, 23 out of 90 samples were tested positive. While, 67 out of 90 samples revealed negative. The sample showing concentration ±>250 pg/ml was considered a positive result.

9.0 Discussion

A total of 90 bat samples were tested for ELISA, and 23 out of them tested positive since the concentration was above 250 pg/ml, as shown in Table 2. The samples tested positive were obtained from Sekayu (Terengganu), Gunung Reng (Kelantan), and Merapoh (Pahang). Hence, SARS-CoV-2 was detected in this study using the ELISA nucleocapsid for viral identification.

However, 67 out of 90 samples were tested negative, indicating low prevalence of SARS-CoV-2 in bats in East Coast Malaysia. The result revealed negative could be due to the sample collection approach. The bats were taken based on random sampling method, which means the bats were chosen randomly as samples. It could be during the sampling, the bats that were taken have not been affected with SARS-CoV-2 yet. Hence, it shows negative results on ELISA viral identification.

Based on the result in Table 1, the value O.D.450 of 0 concentration of the standard solution was 0.217, which was quite relatively compared to other concentration. Supposedly, the value should be the lower since the concentration of the standard solution is 0 mg/ml. Preparation and handling error could be one of the reasons related for this problem. It could be during washing procedure, the plate was not fully empty or clean and did not wash by the Wash Buffer properly. The reason is because the plate was manually washed, which the pipette was used to fill the Wash Buffer into the plate, instead of using automatic microplate washer.

Bat CoVs are widespread over the world and exhibit a high level of genetic diversity, accounting for nearly 60% of all known *Alpha-* and *Betacoronavirus* species, which are thought to have the potential to spread to humans and other mammals, resulting in another SARS-like epidemic (Li et al., 2020).

Based on the results that shows 23 out of 90 samples were tested positive, which proved that SARS-CoV-2 is also present in bats from East Coast Malaysia. Hernández-Aguilar's study had published evidence that more than 200 coronaviruses had been detected in bats from Africa, Asia, Americas, and Europe in 2019. The research has been conducted on the prevalence of SARS-CoV-2 in Thailand in the south (Luo et al., 2022). Thailand is the closest location to Malaysia, contributing to the detection of SARS-CoV-2 in bat samples from East Coast Malaysia.

As stated in the procedure part, the pooling sample was conducted. This method is also related to the positive result shown in Table 2. There is possibility that both of the samples presence of SARS-CoV-2 antigen, however it could be that only one of them had a SARS-Cov-2 which then can contributing to the positive result of the ELISA due to presence of the virus.

ELISA is one of the sensitive method that can be performed in identifying the SARS-CoV-2 in bats. ELISA has a specificity of 97.9%, which is equivalent to previous tests of the same format (Tozetto-Mendoza et al., 2021)

Based on Tong 2022, bats have been repeatedly identified as a natural host for lyssaviruses and, more recently as a possible reservoirs for new human infections such as the Ebola, Marburg, Nipah, and Hendra viruses, as well as SARS-CoV (Tong et al., 2022). Hence, this study is conducted to create awareness of the possibility of coronavirus infection from bats to human. It is proved that bats are one of the reservoir in transmitted the SARS-CoV-2 in human.

However, further confirmation by using real time RT-PCR which is the gold standard method for SARS-CoV-2 detection should be conducted. The study was done where it stated that the COVID-19 outbreak has resulted in an unprecedented global demand for virus infection laboratory diagnostic procedures, and PCR analysis for genomic RNA of SARS-CoV-2 in extracts of swabs collected from the upper respiratory tract has proven to be particularly useful for early diagnosis of infection (Thudium et al., 2021)

10.0 Conclusion

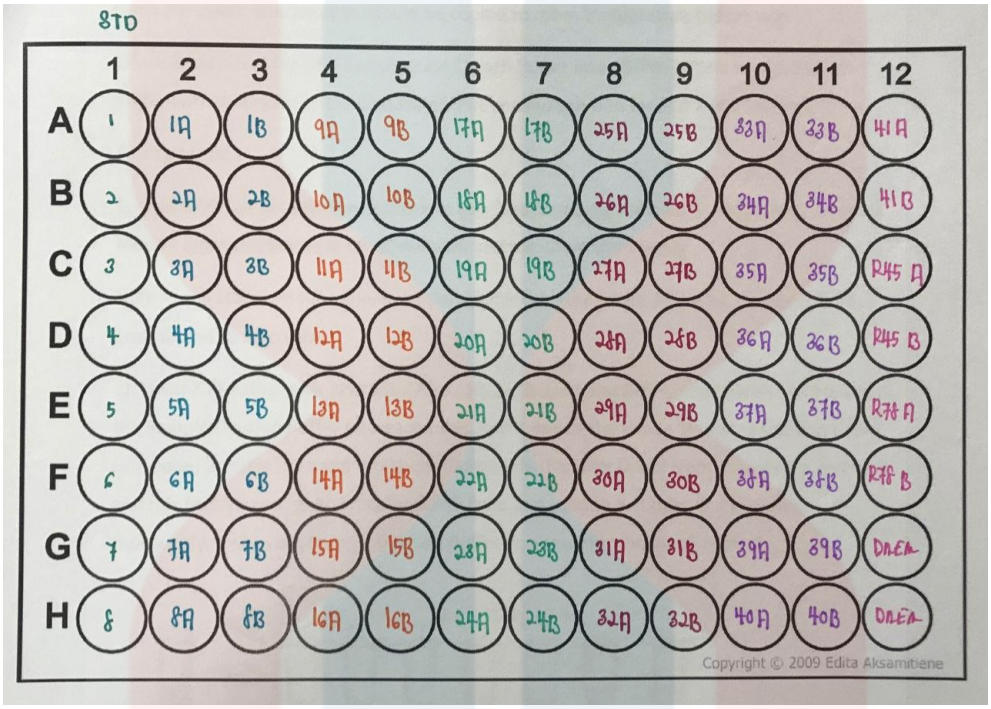
In conclusion, ELISA for SARS-CoV-2 showed positive results in 23 out of 90 samples. Hence, it is proved that there is presence of SARS-CoV-2 in bat samples from East Coast Malaysia. However, further investigation using gold standard method should be done to confirm the result.

11.0 Recommendations and future work

To acquire a better result in future studies, proper preparation and handling should be implemented to prevent the error of the result. It is recommended to used automatic microplate washer in future to make sure the sample is completely being washed hence maintain the accuracy of the result in O.D.45 measurement.

Apart from that, it is recommended to further confirm the result with the gold standard method real time RT-PCR which we cannot conduct it due to the time constraint.

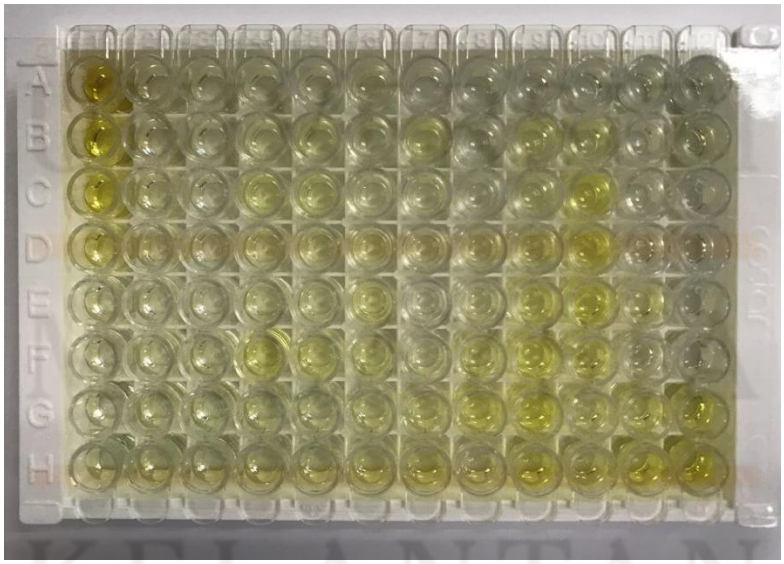
Appendix A



Appendix A: Layout for platting ELISA

Appendix B

Appendix B.1: Standard solution in Eppendorf tubes



Appendix B.2: ELISA plate after addition of stop solution

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