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Original Article

The Existence of *Leptospira interrogans* on Rats and The Transmission Potency in Public Areas: School, Traditional Market, and Settlement in Yogyakarta

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ABSTRACT

Leptospirosis is a zoonotic disease caused by bacterial infection, Leptospira interrogans. Indonesia is known for being an endemic country of this disease and Yogyakarta Special Province has become one of the regions with high cases of leptospirosis. There was lack of information on the L. interrogans prevalence on rats at the public areas, such as school and traditional market. This research was conducted to determine and predict the potential leptospirosis transmission in public areas, especially in schools, traditional markets, and the settlement of Yogyakarta. Wild rats were collected from several public places (elementary schools, traditional markets, and Settlement areas) by using single live traps. The rat's blood was centrifuged to obtain the serum. The serum was tested by using immunochromatography of Leptotek Lateral Flow. The collected rats and shrews were euthanized and then identified for the species and the morphological features. Total of 27 rats (67.5%) and 13 (32.5%) shrews were collected. There were six species of collected rats, namely Rattus argentiventer, Rattus norvegicus, Rattus tanezumi, Rattus tiomanicus, and Bandicota bengalensis, while the collected shrew species was Suncus murinus. The rats and shrews from traditional market were negative with L. interrogans, however the positive result was in elementary schools (14.28%), that were from R. norvegicus and S. murinus, moreover the positive infection also showed in the settlements (57.14%), that were from R. argentiventer, R. norvegicus, and R. tiomanicus. These findings indicated that school and settlement must be a concern for the leptospirosis transmission.

Keywords: Leptospira interrogans; rats; school, settlement, traditional market, Yogyakarta.

Highlights: The novelty in this research was the potency of *Leptospira interrogans* transmission in public areas: school, traditional market, and settlement, as there was limited information on prevalence of infected rats in public areas.

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INTRODUCTION

Leptospirosis or Weil's disease is a zoonotic disease caused by bacterial infection from *Leptospira interrogans*, known as pathologic bacteria with spiral morphological features. Leptospirosis mostly can be found in tropic and subtropic areas, and Indonesia is one of the endemic countries of the disease. This disease can be transmitted to humans and animals through reservoirs such as rats, mice, and shrews.[1,2]

Leptospirosis can be a serious problem but is still treatable with the right treatments. The symptoms of leptospirosis in humans are quite similar to other common diseases such as influenza and hepatitis. The similarity of the symptoms caused this disease to be overlooked by many people.[1] Mild symptoms of leptospirosis are fever, severe headache, sore muscles, diarrhea, and mild jaundice or icteric. Severe symptoms of leptospirosis are severe icteric or jaundice, kidney failure, bleeding manifestation, and anuria.[2]

The main transmission of this disease in humans mostly involves direct and indirect contact between *L. interrogans* infected urine and human skin. Direct transmission of *L. interrogans* can occur when human skin contact directly with infected urine or blood. Meanwhile, indirect transmission of this bacteria can occur when human skin contact with water, soil, or another medium that is contaminated by infected urine or blood.[2,3] Reservoirs of leptospirosis commonly known are animals like mammals, especially rodents. Leptospirosis in Indonesia is mostly caused or transmitted by rodents as reservoirs.[4] Rodents in Indonesia that usually become reservoirs of leptospirosis are *B. indica, R. norvegicus, R. exulans, Mus musculus,* and *Suncus murinus*.[2,4]

The Health Profile of Indonesia in 2016 from The Indonesian Ministry of Health shows that Indonesia is an endemic country of leptospirosis shows that Special Region of Yogyakarta is one of the regions in Indonesia with a high number of leptospirosis cases.[5] The Special Region of Yogyakarta had the highest number of leptospirosis cases in 2010 with 230 cases with 23 death cases.[6] Bantul Regency had the highest number of cases with 154 cases than other areas, yet Yogyakarta City had the highest Case Fatality Rate of leptospirosis.[7]

Diagnosis for leptospirosis can be conducted through IgG and IgM detection in the blood with Rapid Detection Test (RDT) using Lepto Tek Lateral Air Flow.[8] Diagnosis for leptospirosis also can be detected through IgG and IgM detection in Reservoir's blood. In previous research by Romadhona (2022), rats were collected from Settlement areas in four districts of Yogyakarta City (Wirobrajan, Tegalrejo, Kotagede, and Umbulharjo), and the blood's serum was analyzed using Lepto Tek Lateral Air Flow. Positive results were shown in 3 of the 29 rats collected from four districts.[9]

Information about the potential transmission of *L. interrogans* in public areas in Yogyakarta other than Settlement areas is still limited compared to the probability of people in Yogyakarta doing activities there and can be direct or indirect contact with the infected rat's urine. In this research, the public areas that are chosen as the focus of studying *L. interrogans* transmission in elementary schools, settlement, and traditional markets of Yogyakarta.

Elementary schools are chosen as representatives of potential studies about *L. interrogans* transmission in the school environment, considering children in elementary schools age are prone to disease and have crucial growth and development.[10,11] Considering the importance of children's health in schools, the study about the potential transmission of threatening diseases such as leptospirosis should be considered to be needed.

Traditional markets were also chosen as representatives of public areas, for this place become the center of people to do daily transactions for daily goods, such as fresh

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foods. Traditional markets need some criteria for safety in health issues, such as good sanitation, to prevent some infectious diseases from emerging there.[12] Some traditional markets such as Demangan and Giwangan in Yogyakarta are still categorized as not healthy management.[13] Those issues can lead to environmental health problems such as transmission and infectious disease emergence in traditional markets. Furthermore, an unclean environment is liked by the rats, the reservoirs of *L. interrogans*.[14]

Therefore, this research was conducted to determine and predict the potential leptospirosis transmission in public areas, especially in schools, settlement, and traditional markets of Yogyakarta.

MATERIALS AND METHODS

Materials and Tools

Materials used in this research were collected from rats and shrews from public areas in Yogyakarta such as state elementary schools (Serayu, Sinduadi Timur, Karangwuni 1, Pogung Kidul), traditional markets (Demangan and Kranggan), and Settlement area (Sharehouse at Kocoran and Wirobrajan), labeling stickers, rats bait (dried fish, fried tofu, cheese, bread, food waste, etc.), Ketamine HCl, alcohol 70% and 96%, Rapid Test Kit Lepto Tek Lateral Air Flow (*Leptospira* IgG/IgM) from SD BIOSENSOR (Korea Selatan), assay diluent.

Tools that were used in this research were individual or single live traps, cloth sacks, digital scales, rulers, identification keys, sectioning kit, 1 ml and 3 ml syringe, EDTA venoject, microtube, mikropipet, and refrigerated centrifuge.

Methods

This research was using experimental analysis of wild rat's collection and detection of *L. interrogans* from the collected rats and shrew bloods, and descriptive analysis for morphometrical analysis, identification, and potential study of leptospirosis transmission.

Wild Rats and Shrews Collection

Rat's collection methods in this research were modified from basic methods for collection from instruction in The Indonesian Ministry of Health and collection methods from Ristiyanto's.[15,16] Rats were collected from four state elementary schools (Serayu, Sinduadi Timur, Pogung Kidul, Karangwuni 1), two traditional markets (Demangan and Kranggan), and two settlements (Kocoran and Wirobrajan). The traps were prepared using dried fish, fried tofu, cheese, or food waste as bait. The traps were installed in the afternoon and evening of each location with a minimum distance of 5 meters between traps, and then collected on the next day in the morning.

Blood Collection and Serum Extraction

Blood collection were conducted by using the modified method from the Indonesian Ministry of Health (2015) and collection methods from Ristiyanto's.[15,16] The rats in the live traps were put into cloth sack then the rats is released in the cloth sack, and it were anesthetized by using Ketamine HCl 50-100 mg/kg through intra muscular. The anesthetized rats were taken out from the sack and the blood collection conducted through cardio with using 3 ml syringe then transferred into EDTA Venoject.

The serum extraction is carried out with using a refrigerated centrifuge. The blood is transferred into a 1 ml microtube and centrifuged for 15 minutes at 3000 rpm. The collected serum was used in Rapid Detection Test in Lepto Tek Lateral Air Flow.

Rapid Detection Test with Lepto Tek Lateral Air Flow

Widiastuti and Jati[8] conducted whole blood detection of Leptospira IgG/IgM by using Lepto Tek Lateral Air Flow, while in this research was used the serum and whole blood as the materials for detecting *Leptospira* test. The 10 μl serum was inserted

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into the sample well of the kit and then 3 drops of assay diluent were inserted into the buffer well. The result came out after 15 minutes and not longer than 30 minutes. The indicators in this kit consist of three categories; C (Control), G (IgG), and M (IgM). The negative result was when the stripe showed in the control indicator. Meanwhile, the positive result was when the stripe showed in either G or M, or both indicators. The stripe or smear in the G indicator showed the blood or serum sample was positive with *Leptospira* IgG, meanwhile, the stripe or smear in the M indicator showed the sample was positive with *Leptospira* IgM.

Identification of Collected Rats and Shrews

The Identification of wild rats and shrews were based on the identification keys book by The Indonesian Ministry of Health and Pinardi's.[15,17] Identification was carried out by using scales to weigh the rats and shrews. The quantitative morphology measurement was carried out by using a ruler to measure total body length (TL), head-body length (HB), tail length (T), head length (H), hind foot length (HF), and ear length (E). The qualitative morphological observation was carried out by describing the dorsal and ventral fur features, the shape of snout and body, and dorsal and ventral features of the tail.

Trap Success

The success of catching rats in an area was expressed as a successful trap. The trap success was calculated by using the formula[15]:

$$\text{Trap success} = \frac{\text{Number of rats caught}}{\text{Number of rat traps}} \text{ X } 100\%$$

Prevalence of *Leptospira interrogans* in Collected Rats

Prevalence of *L. interrogans* was calculated from the positive results of Lepto Tek Lateral Air Flow compared to the total sample of Lepto Tek Lateral Air Flow test.

RESULTS AND DISCUSSION

Identification, Distribution, and Trap Success of Collected Rats and Shrews

Table 1 showed that there were six kinds of species collected from elementary schools, traditional markets, and settlement areas in Yogyakarta. Those species were *R. argentiventer, R. norvegicus, R. tanezumi, R. tiomanicus, Bandicota bengalensis,* and *S. murinus*, which *R. norvegicus, R. tanezumi,* and *S. murinus* were found at three locations, while *R. argentiventer* and *R. tiomanicus* was only found at settlement. *B. bengalensis* was found at both elementary schools and traditional markets.

R. argentiventer, known as ricefield rat, was identified for having yellowish brown dorsal pelage and broken white ventral pelage.[18,19] *R. norvegicus,* known as Norway Rat or Brown Rat, has a long cylindrical body with total length of more than 350 mm and a blunt conus snout. *R. norvegicus* has a rough texture and greyish-brown colored dorsal and ventral pelage.[18,20] *R. tanezumi,* known as House Rat, was identified for having a rougher and glisten pelage than *R. norvegicus,* and having a yellowish brown color for the dorsal and ventral pelage.[18,21] *R. tiomanicus*, known as Shrub Rat or Tree Rat, was identified for having a greyish-brown dorsal pelage with a broken white or cream-colored ventral pelage.[18] *B. bengalensis* was identified for having typical black-colored dorsal and ventral pelage with rough texture pelage.[18] *S. murinus*, known as House Shrew, was identified as having distinctive quantitative and qualitative morphology differences. *S. murinus* typically has sharp snout with very short tail compared to other species collected from three locations. This species has pungent and distinctive body odor.[21] *S. murinus* has a

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smooth and short pelage compared to other collected species.[22]

Morphometric measurement of the species collected from two or three locations was compared, especially based on total length which can be a typical analysis for size comparison. The variance in the total length of each species showed a particular trend that the rats and shrews species caught from traditional markets were bigger and longer than the species caught from elementary schools. The distinctive difference in size was shown in *R. norvegicus, B. bengalensis,* and *S. murinus* (Table 1). Morphometric differences between some species collected from traditional markets and elementary schools, especially the total length and the weight, can be affected by adaptation to the environment, which was related to the activities of the rats and shrews in eating patterns and habits.[23]

Table 1. Range of Body Weight and Morphometry of Collected Rats and Shrews from Elementary Schools, Traditional Markets, and Settlement in Yogyakarta

Morphometry	Elementary Schools	Traditional Markets	Settlement
	Rattus argentiventer		
W (g)	-	-	64 - 137
TL (mm)	-	-	264 - 322
HB (mm)			139 - 151
T (mm)	-	-	125 - 171
HF (mm)	-	-	34 - 40
E (mm)	-	-	18 - 15
	Rattus norvegicus		
W (g)	207 - 287	292 - 314	237- 298
TL (mm)	365 - 400	405 - 420	396 - 401
HB (mm)	188 - 213	229 - 226	204 - 216
T (mm)	176 - 187	175 - 194	180 - 197
HF (mm)	41 - 43	40 - 44	42 - 45
E (mm)	20 - 21	19 - 22	19 - 21
	Rattus tanezumi		
W (g)	24 - 169	87	116
TL (mm)	222 - 359	284	324
HB (mm)	97 - 183	138	156
T (mm)	125 - 190	146	168
HF (mm)	25 - 35	36	38
E (mm)	15 - 22	20	18
	Rattus tiomanicus		
W (g)	-	-	167
TL (mm)	-	-	358
HB (mm)	-	-	188
T (mm)	-	-	17
HF (mm)	-	-	37
E (mm)	-	-	19
	Bandicota bengalensis		
W (g)	86 - 270	283	-
TL (mm)	285 - 395	405	-
HB (mm)	145 - 205	222	-
T (mm)	140 - 190	183	-
HF (mm)	35 - 40	42	-
E (mm)	18 - 20	21	-
	Suncus murinus		
W (g)	27 - 50	40 – 63	44
TL (mm)	185 - 210	188 – 213	188 - 202
HB (mm)	109 - 119	116 – 140	117 - 126
T (mm)	68 - 91	63 – 83	71 - 76
HF (mm)	18 - 21	18 – 30	20 - 30
E (mm)	9 - 10	6 – 10	8 - 9

Abbreviation: W: Weight; g: gram; TL: Total body length; mm: millimeters; HB: Head-body length; T: Tail length; HF: Hindfoot length; E: Ear length

Table 2 showed the distribution and trap success in elementary schools, traditional markets, and settlement areas. The trap success of the rats was ideal to analyze the density of the rats in a certain area.[24] It was showed that rats can be found in all locations,

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which indicates that there was a rat population at that location. The highest trap success was found in the traditional market at 23.3%. The trap success of rodents such as rats is categorized into high density if the number is higher than 7%.[24]

Table 2. The Distribution and Trap Success of Collected Rats and Shrews

Species	Location			Total (%)
	ES	TM	S	
R. argentiventer	0	0	4	4 (10%)
R. norvegicus	3	4	2	9 (22.5%)
R. tanezumi	7	1	2	10 (25%)
R. tiomanicus	0	0	1	1 (2.5%)
B. bengalensis	2	1	0	3 (7.5%)
S. murinus	3	8	2	13 (32.5%)
Total	15 (37.5%)	14 (35%)	11 (27.5%)	40 (100%)
Trap success	**8.82%**	**14.0%**	**13.75%**	**11.43%**

Abbreviation: ES = Elementary Schools; TM = Traditional Markets; S = Settlement

Traditional markets were a public space known for their unclean condition and for producing food waste. As Saragih et al also Wijayanti and Marbawati said that those conditions were suitable for rats to live in, because rats mostly live in an unclean location and near the food source.[14,25] The number of trap success at traditional markets in this research was higher than the trap success in other research conducted at several traditional markets in Semarang, such as Simongan, Jatingaleh, and Kedung Mundu Traditional market. The trap success at Simongan Traditional Market was 7.0%, the trap success at Kedung Mundu Traditional Market was 4.66%, and the trap success at Jatingaleh Traditional Market was 8.67%.[26,27]

The trap success in settlement in this research was 13.75%, and it was categorized as the high density of rats because it was higher than 7%.[24] Settlement areas that were chosen as the sampling location was sharehouse in Kocoran and Wirobrajan. The trap success at settlement in this research was slightly lower than the trap success in previous research at settlement in urban areas in four District of Yogyakarta. The trap success in four Districts, such as Wirobrajan, Umbulharjo, Kotagede, and Tegalrejo, was about 14.5%.[9] The difference in successful traps was probably due to the number of traps installed, the presence of rats in the area, as well as the density of the human population.

The lowest number of trap success was shown in Elementary schools, it was 8.82%. This number was higher than 7% and categorized as high density of rats.[24] This finding showed higher rat's population than in the previous research that conducted in Krapyak Islamic Boarding School of Yogyakarta. The trap success at Krapyak Islamic Boarding School was 5.9%[28], in contrast the trap success of this research in school was almost one and a half times. The difference in trap success between boarding schools and elementary schools in this research was the existence of residences within the school area may be the biggest factor of increasing the trap success in boarding school more than elementary schools as the sampling location in this research. Residence that close to the school could increase the food source of the rats in the school area. This condition was also supported by the rats caught from elementary schools, that were mostly collected from the school guard's home within the school area, which it can be correlated with the fact about residence for increasing the food source of the rats.[29]

The trap success of rats in each location can be affected by some factors, such as the quality of the live traps, the bait used, the rat's habit, and the location of the trap.[30] The right position of the installed rat trap would made a higher probability of trap success, because of the rat's thigmotaxis behavior which was explaining the behavior

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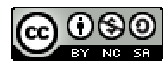

of the rats to always go through the same track.[31]

R. tanezumi was the most caught species in this research. The distribution of the *R. tanezumi* in this research was 7 individuals caught from elementary schools, an individual caught from traditional markets, and 2 individuals caught from settlement. *Rattus tanezumi* was mostly found in elementary schools because *R. tanezumi* is a commensal Rat that is found in a house or buildings in Settlement.[32] *Rattus norvegicus* was the second most caught species in this research with a total number was 9 individuals. *Rattus norvegicus* was mostly caught from traditional markets for 4 individuals. This number was corresponding with the other research results about *R. norvegicus* is the most caught species in Simongan Traditional Market of Semarang.[26]

The factors that affecting the trap success of *R. norvegicus* were the habitat of *R. norvegicus* was at in a bad sanitation locations or in waterways, which was considered as the characteristic of some traditional markets in Yogyakarta.[13,18]

A number of 6 rats out of 33 rats that were caught showed positive for *L. interrogans.* and the prevalence of *L. interrogans* on the each species samples were showed in Table 3.

Table 3. Prevalence of *Leptospira interrogans*

Species	Positive of *L. interrogans* infection (n/N)*			Prevalence in Each Species (%)
	ES	TM	S	
R. argentiventer	(0/0)	(0/0)	(2/3)	66.67%
R. norvegicus	(1/3)	(0/4)	(1/2)	22.22%
R. tanezumi	(0/7)	(0/2)	(0/1)	0%
R. tiomanicus	(0/0)	(0/0)	(1/1)	100%
B. bengalensis	(0/2)	(0/1)	(0/0)	0%
S. murinus	(1/2)	(0/5)	(0/0)	14.28%
Total	(2/14)	(0/12)	(4/7)	**18.18%**
Prevalence in Each Location (%)	14.28%	0%	57.14%	

Abbreviation: ES = Elementary Schools; TM = Traditional Markets; S = Settlement*(n/N) = the number of positive samples/number of samples examined

Table 3 showed the prevalence of *L. interrogans* based on the species and the locations. The total prevalence of *L. interrogans* from all collected blood samples was 18.18%. The previous research by Astuti showed that the prevalence of *L. interrogans* from four District of Yogyakarta City was 10%.[9[in review]] Meanwhile, the previous research by Joharina et al (2019) showed the prevalence of *L. interrogans* in Bantul Regency was 20.4%.[33] The prevalence of *L. interrogans* in this research was higher than Romadhona's research but a little lower than Joharina's.[9,33] This research was conducted at several areas of Yogyakarta City and Sleman, thus can be said that prevalence of *L. interrogans* in several areas of Sleman and Yogyakarta City was higher than in four District of Yogyakarta City, but lower than in Bantul Region. The high result in prevalence of *L. interrogans* from this research can be considered as the presence of more *L. interrogans* in the collected rats and lead to higher potential transmission of leptospirosis.[33]

The prevalence of *L. interrogans* based on the location was showed that positive result was shown in the rats and shrews from elementary school and settlement, but there were negative results in all samples from traditional markets. The prevalence of *L. interrogans* in the rats was heterogenous based on the microhabitat of sampling location.[34] The positive results of *L. interrogans* from settlement and elementary schools was affected by the environment of the locations.

Based in Table 3, the prevalence of *L. interrogans* in settlement was 57.14% and 14.28% in elementary school. The prevalence in the settlement was shown four times higher than the prevalence in school. This can be assumed that the *L. interrogans* transmission in settlement is much easier to occur than the elementary schools. The transmission of *L. interrogans* can occur by direct contact between human skin and the *L. interrogans* infected urine or by indirect contact between

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human skin and the soil or water contaminated by infected urine from the reservoirs.[2,3]

Thibeaux[35] stated about the suitable environment for *L. interrogans* was water and soil near the banks of rivers or water bodies, and this statement was correlated with the locations which was having the humid environment affected by the distance between water ways or water bodies.[35] The location of *L. interrogans* infected rats in the elementary school was found at Sinduadi Timur State Elementary School and Serayu State Elementary School. Sinduadi Timur State Elementary School is located 220 m from Selokan Mataram waterways and is also located around a residential area. Meanwhile, Serayu State Elementary School is located 550 m away from the Code River and located away from the settlement. The location of *L. interrogans* infected rats in the Wirobrajan District which is located 100 m from Winongo river.

The transmission of *L. interrogans* is considered to be correlated with the species of the rats and shrews. Cosson stated that the main host of the *L. interrogans* is the *Rattus* genus.[36] Ikawati's research stated that *R. norvegicus* and *R. tanezumi* have a higher probability of infection than *Suncus* species. *Rattus norvegicus* have almost 78-fold to be infected by *L. interrogans* compared to *Suncus.* Meanwhile, *R. tanezumi* is 8-fold to be infected by *L. interrogans* compared to *Suncus*.[37]

The results of this research showed that the *L. interrogans* infected rats and shrews species was *R. argentiventer, R. norvegicus, R. tiomanicus,* and *Suncus murinus.* The highest prevalence was 100% from *R. tiomanicus* (Table 3). It was shown that this prevalence was higher than the prevalence showed in Joharina's research at Bantul Region with 31%.[33] The prevalence of *L. interrogans* from *R. tiomancus* showed as the highest prevalence because the higher rate of infection in certain area will affect the transmission of *L. interrogans* easier to occur between or within the rat species.[38] *Rattus argentiventer* and *R. norvegicus* is living in the humid area, which had high potential of *L. interrogans* transmission.[39] The prevalence of *R. norvegicus* in this research was 22.22%. It was lower than the prevalence in Joharina's research in Bantul Region with 43%, but it was higher than the prevalence in Sunaryo and Priyanto's with 12.5%.[33,38] Meanwhile, the prevalence of *L. interrogans* from *R. argentiventer* was 66.67%, and it was higher than the prevalence in Ramadhani and Widiastuti's reasearch with 7.69%.[40] The prevalence of *L. interrogans* from *S. murinus* in this research was 14.28%. It showed that the prevalence in this research was higher than the prevalence in Ikawati's research with 1.6% and Ramadhani's research with 6.7%.[36,41]

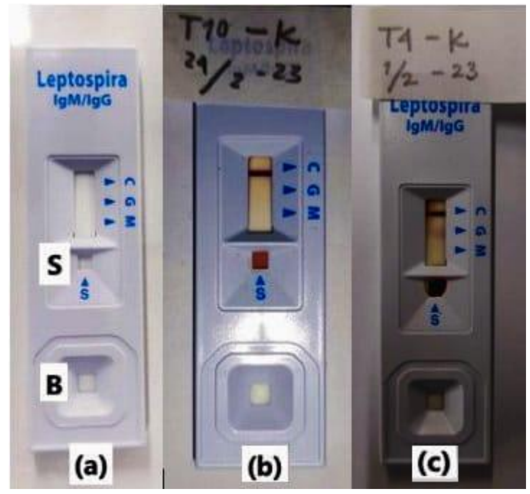

Figure 1. Lepto Tek Lateral Air Flow Kit with C, G, and M indicators (a), Sample well (S), and Diluent Assay Well (B). The Negative result showed in (b) and the positive result showed in (c).

Test results with leptotek showed that in samples rat's that were positively infected with *L. interrogans*, they appeared a line on the IgG and/or IgM line (Figure 1). Table 4 showed the IgG and IgM detection in Lepto Tek Lateral Air Flow from the blood samples. In total of 66.67% and 33.33% of rat's serum were positif with IgG and IgM respectively. The positive results from elementary schools were shown by IgG detection in *R. norvegicus* (50%) and IgM detection in *S.*

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murinus. The positive results from settlements were shown by IgG detection in *R. norvegicus*, *R. argentiventer* , and *R. tiomanicus*, and also IgM detection in *R. argentiventer*.

Table 4. IgG and IgM Detection in Lepto Tek Air Flow from *L. interrogans* infected Rats

Location	Species	Indicator		
		Control	IgG	IgM
Elementary Schools	*R. norvegicus*	+	+	-
	S. murinus	+	-	+
Settlement	*R.argentiventer* I	+	+	-
	R.argentiventer II	+	-	+
	R. norvegicus	+	+	-
	R. tiomanicus	+	+	-
			66.67%	33.33%

The IgM detection in the samples indicates the acute phase of early-stage infection in the reservoir. The IgM detection can be detected in the first two months of infection. The IgM level was appearing earlier than IgG and will quickly be followed by IgG. The IgG detection from the samples indicated the secondary immune response after early detection and can be categorized as the chronic stage of *L. interrogans*.[1,42]

The presence of rats is important, because apart from carrying *L interrogans* bacteria that cause leptospirosis, they can also transmit plague, that it caused by bacterial infection, *Yersinia pestis*, and both of the diseases are zoonotic.[43] A healthy lifestyle, with a clean environment is one of the factors to avoid the presence of rats in our environment and prevent leptospirosis transmission

STRENGTH AND LIMITATION

The strength of this study was that the information regarding the prevalence of bacteria *L. interrogans* in wild rats in public areas, especially schools and traditional markets, is still very limited, and this research can be a starting point and a reference for other researchers. The limitation of this study was the need for exploration and integration of data from leptospirosis patients to provide more description and evaluation of risk factors for leptospirosis in humans.

CONCLUSIONS

Six blood samples from collected rats and shrews from settlement and schools were confirmed positive of *L. interrogans.*, while there was negative results of rats from traditional markets.The prevalence of *L. interrogans* in settlement was 57.14% and at the school was 14.28%. These findings revealed that school and settlement must be a concern for the leptospirosis transmission.

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ETHICAL CLEARANCE

The research protocol was approved by the Research Ethics Committee, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (Reference number 092/EC-FKH/Eks./2022 and 094/EC-FKH/Eks./2022).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in this research.

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AUTHOR CONTRIBUTION

Raden Roro Upiek Ngesti Wibawaning Astuti (RRUNW), Salsabila Rifda Yuangga (SRY), Fahrunniam (FN). Conceptualization: RRUNW. Funding acquisition: RRUN. Methodology: RRUNW, SRY, FN. Original draft preparation: SRY and FN. Writing review, editing and validation: RRUNW.

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Original Article

Diagnostic Test of Blood Eosinophil Level as a Marker of *Ascaris lumbricoides* Infection

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ABSTRACT

A. lumbricoides infection (Ascariasis) is one of 17 neglected tropical diseases in Indonesia. Ironically, many cases of Ascariasis in Indonesia have not been diagnosed properly. This is because stool examination with Kato-Katz's method still rarely done. Therefore, it needs an alternative examination that more simple, easly done and can be routinely used in order to Ascaris diagnosing. This study was a diagnostic test for blood eosinophil levels as a marker in A. lumbricoides infection. This study was conducted in a private hospital at Medan regency. This study involved 63 children in pre-school and school age who had their parent approval. The stool was examined by Kato-Katz method as a gold standard and blood eosinophil levels was examined as an index in this study. The results showed sensitivity level of blood eosinophilia as a marker is 25.00% (CI95%: 5.49-57.19%) and specificity 96.08% (CI95%: 86.54-99.52%). The index also showed positive predictive value 60% (CI95%: 21.93-88.90%), negative predictive value 84.48% (CI95%: 79.63-88.35%), positive likelihood ratio 6.38 (CI95%: 1.19-34.04) and negative likelihood ratio 0.78 (CI95%: 0.56-1.09). The conclusion is elevated blood eosinophil levels cannot be used as an alternative test Kato-Katz in diagnosing Infection of A. lumbricoides. With its low specificity, blood eosinophil does not able to exclude Ascariasis, so it can not be used as a screening. Even though has low specificity, blood eosinophilia has high predictive value that can help practician in order to diagnosing.

Keywords: Eosinofil, A. Lumbricoides, Diagnostic test, Sensitivity, Spesificity.

Highlights: This study aims to find a simple examination to help establish the diagnosis of Ascaris lumbricoides infection. The results of this study indicate that blood eosinophil levels can help establish the diagnosis, but are less sensitive to rule out differential diagnoses.

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Blood Eosinophil Level Count

Blood Eosinophil Level Count is done by measuring absolute blood eosinophil from peripheral blood sample. The sample is indicated as eosinophilia if the increase in blood eosinophil level is more than 0.45×10^3 cells/μL[8,9]. Blood samples that have been taken by experienced phlebotomist are analysed in the hospital laboratory where they are being admitted. The blood was diluted using Dunger's solution with the ratio 1:20, this solution will stain the eosinophil and simultaneously breakdown erythrocytes and leukocytes. After that Improved Neubauer counting chamber will be filled with the solution, let the chamber be for 5 minutes until eosinophil filled and settled inside the counting chamber. This process was done inside a petri dish layered with moisturized absorption paper to prevent evaporation. Under the microscope, eosinophils was counted on four leukocyte square. The calculation must be accomplished before one hour mark to reduce error.

RESULTS AND DISCUSSION

Table 1 shows the result of stool examination using Kato Katz methods. From 63 samples, 14 samples were infected by helminth. Most of them was suffered Ascariasis.

Based on table 2, from 63 samples examined at the hospital where study was held, the number of samples who suffered single *A. lumbricoides* infection occur more frequently on male (7 out of 33 patients (23.3%)) compared to female (5 out of 30 (16.7%)). There is no correlation between patient's sex and *A. lumbricoides* infection due to miniscule difference in lifestyle, behaviour and habit in male and female at this particular age group. It means that both male and female have the same chance to be infected by *A. lumbricoides.* Another study in North Sumatra in 2010 that did not separate A. lumbricoides infections from other soil-borne helminth infections, also showed that there is no correlation between sex and helminthiasis.[11]

Table 2 shows the number of patients who suffered single *A. lumbricoides* infection occurs more in school age group at 6-12 years old (4 out of 18 patients (22.2%)). It is more than patients in pre-school age group (8 out of 45 patients (17.8%)). The number of *A. lumbricoides* infection in this study does not show its correlation with school age children due to adequate sanitation quality in Medan Regency, which cause less soil to be infected by *A. lumbricoides* egg. This situation will make children in those area do not get infected even if they play on the soil.

Table 1. Stool examination result from Kato Katz test.[15]

No	Stool Test Result	Sample (n)	%
1	Single *Ascaris lumbricoides* infection	12	19.0
2	Mixed infection *A. lumbricoides & T. Trichura*	2	3.2
3	Single *T. Trichiura* infection	2	3.2
4	No helminth eggs found	47	74.6
Total		63	100

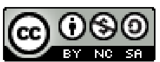

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Table 2. Frequency Distribution of Characteristic and Analysis Result of the Correlation between Sex, Age, Nutritional Status, Blood Hemoglobin Levels and Blood Eosinophil Level

No	Characteristic	Stool examination result using Kato Katz test		N	*p-value*
		Eggs *A. lumbricoides* (+)	Eggs *A. lumbricoides* (-)		
1	Sex				
	Male	7 (11.1%)	26 (41.3%)	33 (52.4%)	0.6463
	Female	7 (11.1%)	23 (36.5%)	30 (47.6%)	
2	Age				
	Pre-school age	9 (14.3%)	36 (57.1%)	45 (71.4%)	0.6848
	School age	5 (7.9%)	13 (20.6)%	18 (28.6%)	
3	Blood eosinophil level				
	Normal	10 (15.9%)	48 (76.2%)	58 (92.1%)	0.0150
	Eosinophilia	4 (6.3%)	1 (1.6%)	5 (7.9%)	

UNICEF's survey in Indonesia showed the number of *A. lumbricoides* infection is higher in pre-school age group (63.7%) than in school age group (53.0%)[10].

Table 2 shows the number of patients who single *A. lumbricoides* infection followed by blood eosinophilia occurs in 4 out of 5 patients (80%). This number is more than Ascariasis patients without blood eosinophilia (10 out of 58 patients (17.1%)). It shows correlation between Ascariasis and blood eosinophilia.

A study taken on elementary student shows helminth infection severity will followed by eosinophil blood elevation.[12] Blood eosinophilia are common in asymptomatic helminthiasis in rural area.[13]

Furthermore, the patient of single *A. lumbricoides* infection taken out to see correlation between single *A. lumbricoides* infection and blood eosinophilia. Based on Table 3, in this study shows that samples with single *A. lumbricoides* infection mostly affect those with blood eosinophilia (3 out of 5 patients) compared to patients without elevated blood eosinophil level (9 out of 58 patients).

Table 3. Blood and Stool test result

Blood eosinophil level	Stool examination result using Kato Katz test		Total
	Single infection *A. lumbricoides*	Mixed Infection with no *A. lumbricoides egg*	
Blood eosinophilia (+)	3 (4.8%)	2 (3.2%)	5
Blood eosinophilia (-)	9 (14.3%)	49 (77.8%)	58
Total	12	51	63

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There is almost no test that qualify as gold standard, that become a reference as comparison in statistic test for other test in parasitology, and no test with 100% accuracy to confirm helminthiasis, including *A. lumbricoides*[14,15].

WHO still recommends *Kato-Katz* test for surveillance and epidemiological survey in *A. lumbricoides* infection because it is relatively simple, fast, cheap and can be classified based on infection severity[16]. This study compares diagnostic test results of elevated blood eosinophil level with Kato Katz test result as a gold standard.

Based on the result in table 3, sensitivity will be calculated using the following formula $\frac{a}{a+c} * 100\%$ a/(a+c)x 100%. Sensitivity of blood eosinophilia as a marker for Ascariasis is 25.00% (CI 95%: 5.49%–57.19%).

Specificity will be calculated with the formula $\frac{d}{b+d} * 100\%$ from the data acquired in table 3. Specificity of blood eosinophilia as a marker for Ascariasis is 96.08% (CI 95%: 86.54%–99.52%).

Positive Predictive Value will be calculated using the formula $\frac{a}{a+b} * 100\%$ from the data acquired in table 3. Positive predictive value for blood eosinophilia as marker to indicate Ascariasis is: 60% (CI 95%: 21.93%–88.90%).

Negative Predictive Value will be calculated using the formula $\frac{d}{(c+d)} * 100\%$ from the data acquired in table 3. Negative predictive value for blood eosinophilia as marker to Ascariasis is: 84.48% (CI 95%: 79.63%–88.35%).

Positive likelihood ratio will be calculated using the formula $\frac{sensitivity}{1-specificity}$ from the data acquired in table 3. Positive likelihood ratio for blood eosinophilia as marker to indicate Ascariasis is: 6.38 (CI 95%: 1.19–34.04).

Negative likelihood ratio will be calculated using the formula $\frac{1-sensitivity}{specificity}$ from the data acquired in table 3. Negative likelihood ratio for elevated blood eosinophilia level as marker to indicate *A. lumbricoides* infection is: 0.78 (CI 95%: 0.56–1.09).

Table 4. Diagnostic Test Result for Elevated Blood Eosinophil Level

Blood Eosinophil Result	Diagnostic Test Result					
	Sens.	Spes.	PPV	NPV	LR+	LR-
	25.00%	96.08%	60%	84.48%	6.38	0.78

Abbreviation
Sens. = Sensitivity
Spes. = Specificity
PPV = Positive Predictive Value
NPV = Negative Predictive Value
LR+ = Positive Likelihood Ratio
LR- = Negative Likelihood Ratio

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According to table 4 result, the sensitivity of elevated blood eosinophil level as diagnostic tool for marking *A. lumbricoides* infection is 25.00% with 96.08% specificity.

The 25.00% sensitivity means that, from observation done to 100 patients infected by *A. lumbricoides* and tested for eosinophilia, only 25 patients can be correctly diagnosed and the remaining 75 patients are failed to be diagnosed although they are really infected. This shows that the number of false negative is high. If there is no elevation in blood eosinophil level (normal blood eosinophil level), this cannot rule out the possibility of *A. lumbricoides* infection.

The 96.08%, specificity means in 100 healthy person that are being tested for blood eosinophil level, about 96 people are correctly indicated as healthy, and about 4 person are indicated as being infected by *A. lumbricoides* even though they are not. This means that the number of false positive is miniscule that it could help in diagnosing *A. lumbricoides* infection if the anamnesis result and physical diagnostic examination support it.

A study in North Argentina, 2012, compared test results of *A. lumbricoides* infection using *Kato-Katz* method, *McMaster* and *Mini-FLOTAC* with flotation salt solution FS2 (NaCl) and FS7 ($ZnSO_4$), using the gold standard the result was positive in one of those test. The result of sensitivity test was 87.1 % for *Mini-FLOTAC* with FS7 solution, *Kato-Katz* 84.4%, *Mini-FLOTAC* with FS2 solution 61.3% and *McMaster* only about 48.3%[17].

A study in Brazil showed that the A study in Brazil showed that the sensitivity of faecal egg count *A. lumbricoides* are respectively 97.3%, 94.2% and 69.5% for *Kato-Katz*, *Formalin-Ether Sedimentation* and *McMaster*[18]. A study in several countries, showed that *Kato-Katz* method is better compared to *McMaster* in faecal egg count for *A. lumbricoides*[19]. Another in Ethiopia show the sensitivity of *Kato-Katz* as a single test is only 67.8%[20].

In meta-analysis that involves many research, the sensitivity of *Kato-Katz* test is various. Sensitivity of *Kato-Katz* test on 1 sample for 1 slide is 63.8%, sensitivity of *Kato-Katz* test on 1 sample for 2 slides is 64.6%, sensitivity of *Kato-Katz* test for 2 samples taken from 1 patient is 69.2%, sensitivity of *Kato-Katz* test for 3 samples taken from 1 patient is 70.4%. This number looks higher if compared to the sensitivity direct microscopic examination (52.1%), *Formol-Ether Concentration* (56.9%) and *McMaster* (61.1%). Sensitivity of *Kato-Katz* test lies under *FLOTAC* and *mini-FLOTAC* (79.7% dan 75.5%)[21].

A study in Philipines around 2004-2005, showed *Kato-Katz* test have good sensitivity and specificity, but this level of sensitivity and specificity may vary from day to another. Around 8.8% samples in that study with *Kato-Katz* test generated change in the result, this can be from negative results to positive result (4.9%) or positive result to negative (3.9%)[15]. Elevated blood eosinophil level in Ascariasis will be more stable because the increase of blood eosinophil level will remain for some time in blood and tissue because the helminths have life cycle that cross the host's tissue[4,8].

Kato-Katz test for faecal egg count specifically for *A. lumbricoides* egg also have weakness. By using *Kato* solution that contain glycerol, hyalin layer will be dried off and causing the inside of the egg to be more visible. But after a few minutes, the hyalin layer may cause distortion and sometimes damage the egg. This will make the egg to be misidentified as another object and reported as no egg were found. This could affect *A. lumbricoides* egg even not as frequent as to hookworm egg[18].

High specificity of blood eosinophil examination level may be useful in diagnosing Ascariasis. But even though the specificity is high, its sensitivity is low. This means that the elevation of blood eosinophil

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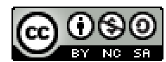

level cannot be used as a tool for Ascariasis screening in a population. Diagnostic test for screening purposes must have high sensitivity even its specificity is quite low[22]

The main objective from a diagnostic test is its utilization to confirm the diagnosis. Sensitivity and specificity are not useful to indicate weather if an individual that being examined suffer from a disease or not based on the test result that being used. That is why a probability degree is needed in a test to diagnose a disease. It is called as predictive value[23]. Positive predictive value is a probability an individual really suffer from a disease if the test result is positive. And negative predictive value is the probability an individual does not suffer from a disease if the diagnostic test result is negative[22].

Table 4 shows the positive predictive value of Blood eosinophilia as a marker for Ascariasis is 60%, and its negative predictive value around 84.48%.

Blood eosinophilia as a marker had 60% positive predictive value means that by observing blood eosinophilia a clinician can convince themself that their patient have 60% possibility being infected by *A. lumbricoides*. 84.48% negative predictive value means a clinician can convince themselves that their patient has 84.48% possibility not being infected by *A. lumbricoides* if they have normal blood eosinophil level.

In contrast with sensitivity and specificity, predictive value is unstable to be used as diagnostic test. Its value is really fluctuative and depends on disease prevalence[22]. This means that predictive value in this study can only be used on another region with equivalent prevalence for *A. lumbricoides* infection.

The last parameter that has been acquired from diagnostic test using data from table 3 is likelihood ratio. Likelihood ratio is comparison of likelihood to get one specific result from a test done on group of infected samples with group of healthy samples[24,25].

Based on the statistic test result on table 4, positive likelihood ratio of blood eosinophilia as marker for Ascariasis is 6.38, and its negative likelihood ratio around 0.78.

Blood eosinophilia as a marker had 6.38 positive likelihood ratio means that the group with Ascariasis have tendency 6.38 times higher to generate elevated blood eosinophil level compared to those without the infection. This number shows the ability of blood eosinophilia to support the diagnosis. Positive likelihood ratio below 10 indicate that the test is not strong enough in confirming the diagnosis[24].

This tool also had 0.78 negative likelihood ratio means that the likelihood of blood eosinophilia in Ascariasis is only 0.78 times compared to samples without the infection (0.78:1). Hence, the group without Ascariasis only have the likelihood of 1.28 times higher not to have blood eosinophilia compared to the group with the Ascariasis (1:1.28). This number shows the weak ability of negative test result from blood eosinophilia in excluding the diagnosis of Ascariasis. A test will able to exclude a differential diagnosis if the negative likelihood ratio is below 0.1[24].

Likelihood ratio is not only used to determine the ability of a test in helping the diagnosis or excluding other possible diseases, but also can be used to calculate the probability of a disease after examinations have been done. To calculate the probability of a disease in a patient after observing the possibility of the test result, a tool called *Nomogram Feye* is needed[24,25].

In Figure 1, simulation using *Nomogram Feye*, shows the likelihood of *A. lumbricoides* infection on pediatric age at the hospital where study was held is initially 19.04% and can increase to 60% if the eosinophil count shows increased level. The likelihood for *A. lumbricoides* infection to occur on pediatric patients decreased to approximately 16% if the blood count does not show any increase in blood eosinophil level. This indicates the usefulness of elevated blood eosinophil level as a marker in supporting the diagnosis of *A. lumbricoides* infection.

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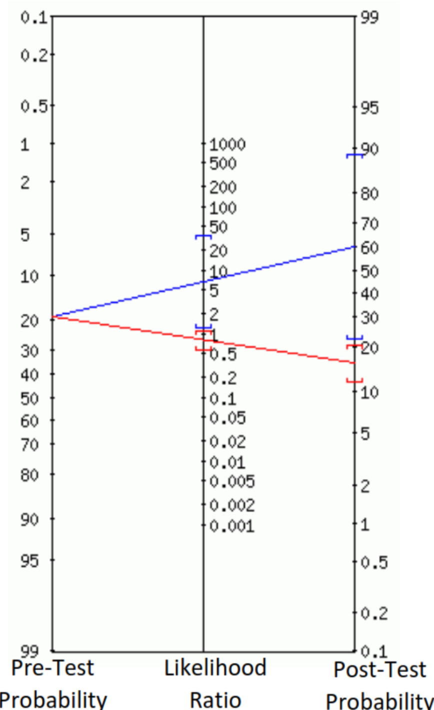

Figure 1. Pre-test and Post-test probability *A. lumbricoides* infection after blood eosinophil level test.

CONCLUSIONS

The prevalence of A. lumbricoides infection on pediatric patients at the hospital where study was held is 22.2%. A. lumbricoides infection on pediatric patients affect more female patient at school age. Pediatric patients with A. lumbricoides infection affect larger number of children with normal nutritional status, children with decreased blood haemoglobin, and children with elevated blood eosinophil level.

Diagnostic test result for elevated blood eosinophil level as an indicator A. lumbricoides infection are: sensitivity 28.57% (CI95%: 8.39-58.10%), specificity 97.96% (CI95%: 89.15-99.95%), positive predictive value 80% (CI95%: 32.67 97.06%), negative predictive value 82,76% (CI95%: 77.47-87.02%), positive likelihood ratio 14,00 (CI95%: 1.70-115.40), negative likelihood ratio 0,729 (CI95%: 0.52-1.02).

Specificity of blood eosinophilia may be useful in diagnostic process, but it shows low specificity on the test, so it is unusable in excluding other differential diagnosis. This low sensitivity cannot be used in Ascariasis screening. High predictive value of elevated blood eosinophil is useful to help clinicians in interpreting the test results and diagnose the patient.

In accordance to sensitivity and specificity, its high positive likelihood ratio and low negative likelihood ratio, show that blood eosinophilia is useful in supporting the diagnosis of A. lumbricoides infection, but not useful enough in excluding other differential diagnosis.

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ETHICAL CLEARANCE

The research protocol was approved by The Research Ethic Committee of Medical Faculty Medicine, University of North Sumatera (KEPK FK USU) by issuing a letter with the number 319/TGL/KEPK FK USU-RSUP HAM/2017.

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CONFLICT OF INTEREST

All authors have no conflict of interest.

AUTHOR CONTRIBUTION

Writer, literature searcher, collecting data from literature: SMR,TRIP,DE, conceptor and supervision :MP, review and supervision: AAD.

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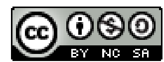

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Original Article

Larvacidal Activity of the Mulberry (*Morus alba* L.) Leaf Extract Against Larvae of *Aedes aegypti*

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ABSTRACT

Dengue Haemorrhagic Fever (DHF) is one of the major public health problems in Indonesia. As the population density increases, the number of sufferers increases. *Aedes aegypti* mosquitoes are vectors for the disease. The absence of drugs make the best prevention effort by eradicating mosquito nests, killing larvae and adult mosquitoes. Mulberry leaves (*Morus alba L.)* may be used as larvacides in the presence of chemical compounds of flavonoids and saponins that inhibit feeding and disrupt the process of insect metabolism. The purpose of this research has to determine the effect of mulberry leaf extract (*Morus alba L.),* to determine the larvicide effect of mulberry leaf extract (*Morus alba L.*) and to determine the concentration of mulberry leaf extract (*Morus alba L.)* which is optimal in killing third instar *Aedes aegypti* larvae. This research used Randomized Design Group (RDG) method with treatment consisted 4 concentrations (0.25%, 0.5%, 0.75%, and 1%), negative control and positive control (ABATE) with 6 repetitions. The results of probit analysis showed that LC_{50} values were 1.124% and LC_{90} was 4.413%. From the one way ANOVA test at each concentration of 0.25%, 0.5%, 0.75%, and 1%,the F count result is 208.331, the value was greater than F table which is 2.53 and the significant value is 0.000 (sig <0.05) then mulberry leaf extract (*Morus alba L.)* has a affected to eliminated of *Aedes aegypti* larvae. Conclusion from the results of the one way ANOVA test of mulberry leaf extract (*Morus alba L.)* was affected to eliminated third instar *Aedes aegypti* larvae.

Keywords: Larvacidal; *Aedes aegypti*; Mulberry Leaf Extract; Vector; Natural Product

Highlights: This research proved the effectiveness of mulberry leaf extract (Morus alba L.) against third instar of Aedes aegypti larvae and has potential as alternative products to synthetic insecticide.

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INTRODUCTION

Indonesia is one of tropical country in the world. Tropical condition cause vector borndisease grow rapidly such as malaria, dengue fever, filariasis, and chikungunya disease. *Aedes aegypti* is a very important disease vector, transmitting the arbovirus causing dengue hemorrhagic fever and chikungunya in human. At present, no effective vaccine is available for dengue, therefore, the only way of reducing the incident of this disease is by controlling the vector, mosquitos, which frequently depends on applications of synthetic insecticides. Eradication of mosquitos borne disease is to break the chain of life cycle of mosquitos that consist four steps; eliminating the cause of the disease, isolation of the patient, preventing mosquitos bite, and vector control[1]. Vector control effort have been carried out various ways that is mechanics, biology and chemistry[2]. However, the use of these chemicals insecticides has enormous negative impact such as environmental pollution, predatory mortality, targeted insect resistance, and causing various dangerous disease in human.

Based on the research of concerning larvae effect from various natural compound, many research showed that saponin and flavonoid from medicinal plans have effect of larvicides[3]. More important fact is the plant extract are sometimes more effective than the synthetic pesticide and phytochemical have the major role in mosquito control programme[4]. In this sense, substances extracted from plants present a great perspective for the control of *Aedes Aegypti* and other vectors of vector born disease.

Many biological effects including free radical scavenging activity have been reported for flavonoids, which are generally attributed to their structural features. The flavonoid content in mulberry leaves was ranging from 26.41 ± 1.14 mg to 31.28 ± 2.12 mg which are effective as larvacide[5]. Some research found the efficacy of using natural product for larvicidal against *Aedes aegypti* larvae, such as *Acacia nilotica, Baccharis reticularia, Bauhinia pulchella*, *Bauhinia ungulate, Cinnamomum osmophoeum, Cunninghamia konishii, Curcuma longa, Eucalyptus camaldulensis, Eucalyptus nitens, Mentha spicata,* and many more species been identified as promising larvacide[6].

Based on this fact, an alternative larvicidesderived from natural compounds needed to reduce the use of chemical insecticides and discoveries of other potential natural product been done based on the active ingredients which impacted the longevity of *Aedes agypti* larvae. This research aimed to determine the larvicide effect of mulberry leaf extract (*Morus alba L.*) and to discovered the efficacy of mulberry leaf extract (*Morus alba L.)* as natural product against *Aedes aegypti* larvae.

MATERIALS AND METHODS

This research was an analytic experimental study in accordance as described by World Health Organization (WHO) guidelines for laboratory and field testing of mosquito larvicides. This study was conducted in the Laboratory of Parasitology Laboratory of Institute of Health Bhakti Wiyata Kediri.

1. Preparation of test materials

Aedes aegypti mosquito eggs were obtained from the Public Health Office of East Java. The larvae were cultured and maintained in the Laboratory at 27ºC and 85% of relative humidity. The mosquito eggs then placed in plastic tray filled with water as for the maintenance of the larvae. Mosquitos' eggs will hatch into larvae within 1-2 days. Hatching eggs into larvae

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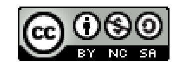

are separated by using larval pipettes for colonization and fed by chicken's liver. After the third phase instar larvae, the larvae are removed by using a larval pipette into a plastic cup containing extract with different concentrations in each cup.

2. Mulberry leaf extract Preparation

Mulberry leaf extract made in accordance with the method of maceration for 24 hour using ethanol 96% as solvent.

Mulberry leaves were purchased from Kayon flower market, Surabaya, Indonesia. After remove any materials and cleaning under tap water, the Mulberry leaves were stored in an oven and dried in the sunlight and then stored at room temperature until further use. The 500 g of the plant sample powdered were soaked in ethanol and chloroform separately for 24 hrs. The maceration product then filtered and concentrated under 40ºC using rotary evaporator and produced 31 ml mulberry extract.

Ethanol extract of mulberry extract dilute by aquadest to 0.25%, 0.5%, 0.75%, and 1%. As for positive control is abate containing 0.01% temephos, and tap water as negative control.

3. Larvicidal Activity of mulberry extract

The larvicidal activity was assesed by the procedur of WHO and Pesticide Commission. According to WHO procedure, concentration is considered to have an effect when causing death test larvae of 10-95% which will be used to find the value of lethal concentration. Meanwhile, according to the Pesticide Commission, the use of larvicides is said to be effective if it can kill 90-100% test larvae.

4. Bioassay Experiment

For the bioassay test, larvae were taken into five batch, 25 larvae *Aedes aegypti* of eachbatch, in 100 ml desired concentration of mulberry extract (0.25%, 0.5%, 0.75%, and 1%). The negative control wastap water and 0.01% temephos as positive control. After the adding the larvae, the glass dishes were kept in laboratory at roomtemperature. The number of larvae death were counted after 24 hours of exposure, and the percentage of larvae mortality was reported from the average of six replicate. Dead larvae were removed as soon as possible in order to prevent decomposition, which may cause rapid death of remaining larvae. The mean of death of each treatment group in each unit of observation time was tested by using Probit analysis until LC_{50} value was obtained.

RESULTS AND DISCUSSION

Plant extracts exert a multitude of biological activities on pests including larvicide, repellent, insect growth regulator, and more[7,8,9]. This may be because different phytochemicals found in plants can work synergistically to induce such reactions. Plant pesticides are biodegradable and rarely become resistance to pests duel to the synergistic action of complex biomolecules, thereby reducing the long-term environmental impacts of their use[10,11].

Several studies found the potential compound from natural products have larvicidal activity. A review conducted by Wuillda *et al*, revealed about 86 compounds were settled as potentially larvicidal, and wide variety of compounds have been found, such as acetogenins, alkaloids, naphthoquinones, lignans, quassinoids, flavonoids, fatty acids, monoterpenes, sesquiterpenes, and others [12].

Roots, bark and leaves of *Morus alba L.* are used for various health benefit and the presence of precious phytochemicals (coumarins, flavonoids, phenols) of *Morus*

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alba L. leaves possess pharmacological importance. Concentrations of total phenolic compounds of *Morus alba L.* like tannins, alkaloids and saponins were within safe range [13].

The 24hr bioassay is major tool for evaluating the toxicity and have been applying by many researcher. The mosquito larvae exposed under mulberry leaf extract showed significant behavioral changes were observed within 30 minutes of exposure. The most obvious sign of behavioral changed was inability to come on the surface, restlessness, and led to death. No such behavioral change were observed in control group.

This research was conducted in Laboratory of Parasitology Laboratory of Institute of Health Bhakti Wiyata Kediri. The result study are presented in the following Table 1 and the analysis was present on Table 2.

Table 1. Mortality Data of *Aedes aegypti* larvae after 24 hour exposure Mulberry Leaf Extract

Concentration (%)	Total Larvae	Repeatition						Mean	Mortality
		1	2	3	4	5	6	X	%
0.25%	25	12	16	14	18	18	18	16	64
0.5%	25	13	14	15	17	17	17	15,5	62
0.75%	25	14	14	16	17	17	18	16	64
1%	25	14	15	14	17	18	18	16	64
Postive control	25	16	16	17	19	18	19	17,5	70
Negative control	25	0	0	0	0	0	0	0	0

Tablel 2. Analysis Probit

Concentration (%)	Percentage of Larvae Death	LC_{50} (%)	LC_{90} (%)
0.25%	64%	1.124% (0.154-1.744)	4.413 (3.613-5.939)
0.5%	62%		
0.75%	64%		
1%	64%		

Result of experiment conducted for evaluating the larvicidal efficacy of Mulberry Leaf Extract showed that is toxic to *Aedes aegypti* larvae. Lethal concentration of mulberry leaf extract were 1.124% (LC_{50}) and 4.413% (LC_{90}). Based on the results of this study, it can be seen that the extract can be used as larvacide. This occurs because the mulberry leaf extract contain active compounds such as alkaloids, soponin, flavonoids and other chemicals that can affect the nervous system, digestion and breathing in larvae[7,14]. Mortality of mosquito larvae showed no big difference value from all concentration,it indicates that the extract is toxic[15,16]. In this study the temperature, pH and humidity are still at normal limits, so the possibility of mosquito larvae in this study died caused by external influences.

Variation of mosquito larvae mortality caused by the variety of

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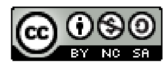

sensitivity and resistance of each larva to the material active in the extract[17,18]. The death of the larvae is caused by the inability of the larvae to detoxify the toxic compounds thatenter the body[19,20]. Based on the results of the observations during the larvae test exhibited anxiety symptoms characterized by upward motion movements on the test medium, while the larvae control showed aresting state on the surface forming angels [16,17,21].

The difference in the percentage of larval mortality is duel to the diffusion speed of extracts entering into different cells so thatat low concentrations the larvae can still tolerate these toxic compounds, whereas at high concentrations the larvae can not tolerate the entry of these toxic compounds[22]. The interaction of toxic substances a biological system is determined by the concentration and length of time. Toxic substances that play a role in lethal larvae are alkaloids, saponins, and flavonoids. Alkaloids that enter the body of the larvae through absorption and degrade the skin cell membrane, besides alkaloids can also interfere with the larva nervous system work[14,15].

Alkaloid compounds act as larvicides by inhibiting the feeding power of the larvae (antifeedant), so the larvae will experience nutritional deficiencies and eventually die[18]. Based on the results of these studies the alkaloids contained in the leaves of elasticity serves as a poison or poisoning stomach. The alkaloid can also be used as an insecticides. The alkaloid compound inhibits the work of acetylcholinesterase enzyme that serves in continuing stimulation to the nervous system, so transmission of excitement does not occur[23,24]. Another active compound contained in the mulberry extract is saponins[25]. Saponins result in decreased activity of digestive enzymes and the absorption of food in insects. In addition, saponins also damage the larvae and causing the death of larvae[3,25,26]. *Morus Alba* extract and its other compounds usually flavonoids have antioxidant properties by scavenging free radicals and protect many organs from oxidative stress [5,13].

CONCLUSIONS

As the leaf extract of Mulberry is toxic for *Aedes aegypti* larvae even at low doses, the plant may eventuallyprove to be useful larvicide. The plant can be eco-friendly and may served as suitable alternative to synthetic insecticides as they are relatively safe, inexpensive and available in many areas of the world.

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CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest.

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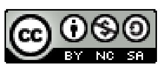

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Original Article

THE INCIDENCE AND CHARACTERISTICS OF MISDIAGNOSED COVID-19 PATIENTS WITH DENGUE FEVER INFECTIONS AT UDAYANA UNIVERSITY HOSPITAL IN 2020-2021

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ABSTRACT

The rise in dengue fever in recent decades combined with the emergence of COVID-19 at the end of 2019, has created new challenges in the healthcare sector. This research is a descriptive study with a cross-sectional research design and using medical record data at Udayana University Hospital in 2020–2021. According to the study, 1.22% cases of misdiagnosis out of a total of 2365 suspected cases of COVID-19 were found at Udayana University. The majority of cases of misdiagnosis involved people older than 60 years, namely 7 people (24.1%) and were dominated by men, namely 17 people (58.6%). The most common symptoms found are fever, cough, shortness of breath, headache, and malaise, According to laboratory results, dominant patients have thrombocytopenia, followed by high alanine transaminase (ALT), high aspartate transaminase (AST), and leukopenia. The appearance of thrombocytopenia in cases of COVID-19 with dengue fever is the result of suppressed platelet synthesis due to virus induction which causes bone marrow suppression and platelet clearance. Leukopenia and leukocytosis may coexist with lymphopenia as an indicator of disease severity. The similarity of symptoms and laboratory results between COVID-19 and dengue fever allows for misdiagnosis that will affect the patient's management. Therefore, the aim of this study is to determine the misdiagnosis rate of COVID-19 with dengue fever at Udayana University Hospital in 2020–2021, so that it can reduce misdiagnosis of the disease.

Keywords: misdiagnosis, clinical characteristics, COVID-19, dengue fever, thrombocytopenia

Highlights: The novelty of this research is that it discusses the emergence of cases of misdiagnosis and the clinical characteristics of misdiagnosed COVID-19 patients with dengue fever at Udayana University Hospital. The benefits of this study are expected to be a reference for future studies regarding the similarity of the clinical manifestations of COVID-19 and dengue fever.

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INTRODUCTION

Coronavirus disease-19 (COVID-19) is an acute respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-COV-2). COVID-19 can cause a series of signs of atypical respiratory disease.[1,2] The outbreak of COVID-19 originated in Wuhan, Hubei Province, China, and spread quickly to various countries.[3] Transmission of SARS-CoV-2 can be through a droplet or an aerosol, causing rapid transmission of the virus.[4] On COVID-19, the reproduction number (R_0) ranges from 1.4 to 6.49.[5] The rapid transmission caused the World Health Organization (WHO) to declare COVID-19 a world pandemic on March 11, 2020.[6]

Dengue Fever is a disease that is transmitted by Arthropod. The vector that causes this disease is *Aedes spp*. especially *Aedes aegypti* and *Aedes albopictus*. Dengue virus has 4 types of serotypes namely DENV-1, DENV-2, DENV-3, and DENV-4.[7–10] This virus serotype is known to have different genotypes, these different serotypes and genotypes will affect the severity of dengue fever.[11] *Aedes spp.* mosquitoes have habitats in the tropical and subtropical regions of the world and have become endemic in several regions. The American, Asian, African, and Australian continents became several regions affected by the dengue fever epidemic.[9]

Dengue infections reported to the WHO have increased significantly over the past several decades, from 505.430 cases in 2000 to 5.2 million cases in 2019. Dengue fever is estimated to have occurred in around 390 million cases worldwide in every years. Around 96 million cases of dengue hemorrhagic fever cause clinical manifestations.[12,13] Meanwhile, COVID-19 has infected more than 650 million people in the world and resulted in more than 6.6 million people dying.[14] The increasing condition of dengue hemorrhagic fever and the emergence of the pandemic COVID-19 raise new challenges to establishing the diagnosis of the two diseases. This is due to the similarity of the symptoms and the disease's laboratories' characteristics. This problem causes challenges in enforcing the diagnosis of the disease.[15,16] Therefore, we researched to find out the incidence and characteristics of misdiagnosed COVID-19 patients with dengue fever infections at Udayana University Hospital in 2020–2021.

MATERIALS AND METHODS

This research is a descriptive study with a cross-sectional research design. The study was conducted in 2020–2021 using medical record data at Udayana University Hospital. The technique used to determine the sample in this study is total sampling. The variables studied in this study were age, gender, complete blood count (CBC), alanine aminotransferase (ALT), aminotransferase (AST), blood urea nitrogen (BUN), serum creatinine (SC), symptoms, and patient conditions. The inclusion criteria of this study are patients with suspected COVID-19. Meanwhile, the patients positively confirmed COVID-19 via real time polymerase chain reaction (RT-PCR) swabs and patients with a fever for 7 days are its exclusive criteria.

Misdiagnosis cases are defined by changes in the diagnosis of COVID-19-suspected patients to a negative diagnosis of COVID-19 and the diagnosis of dengue fever infections. The negative diagnosis of COVID-19 is determined by two negative RT-PCRs. The diagnosis of dengue fever was determined through WHO guidelines, namely fever < 7 days with two of the following: headache, arthralgia, retro-orbital pain, rash, myalgia, hemorrhagic manifestations, leukopenia, and laboratory results such as thrombocytopenia, serum creatine levels, and increased aminotransferases.[17]

Data is processed using the Statistical Package for the Social Sciences (SPSS) for Windows version 25. The study has obtained ethical clearance from the Research Ethics Commission of the Faculty of Medicine,

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Udayana University with number: 531ruN l4.2.2.Vll.14lLT12022.

RESULTS AND DISCUSSION

The total number of cases of COVID-19 in Udayana University Hospital from March 1, 2020, to December 31, 2021, is 2365 and the demographic characteristics has serve on Table 1. Of these, 29 cases (1.22%) were misdiagnosed with COVID-19 with dengue fever.

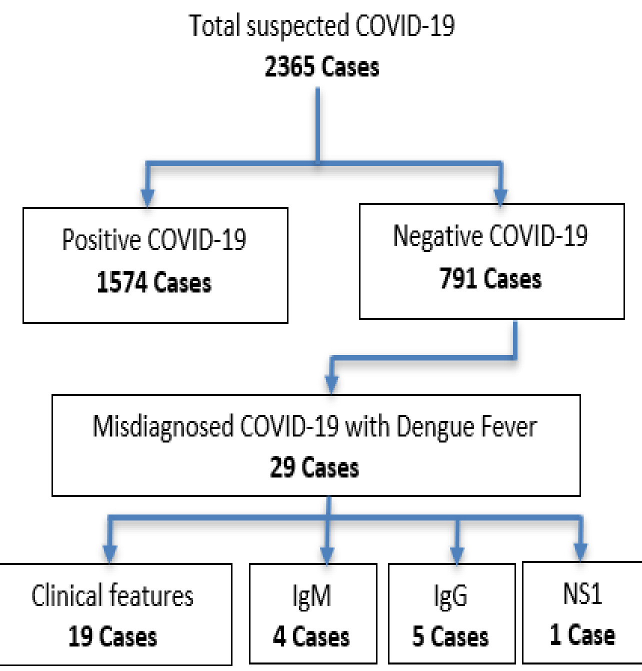

Figure 1. Total Population of Suspected COVID-19

Based on Figure 1. There were a total of 2365 cases of suspected covid-19. Of the total, 1574 cases were diagnosed with COVID-19 via RT-PCR and 791 cases obtained negative results on RT- PCR. Of the COVID-19 negative numbers, 29 cases were diagnosed with dengue fever. Diagnosis of dengue fever was obtained through clinical features of 19 cases, IgM of 4 cases, IGG of 5 cases, and NS1 of 1 case.

Table 1. Demographic Characteristics of Misdiagnosis in COVID-19 Patients with Dengue Fever

Characteristics	n(%)
Age;	
1-10	0(0)
11-20	2(6.9)
21-30	5(17.2)
31-40	5(17.2)
41-50	5(17.2)
51-60	5(17.2)
>60	7(24.1)
Age (years); Average (±SD)	45.69 (± 19.1)
Gender;	
Male	17(58.6)
Female	12(41.4)
Profession;	
Housewife	2(6.9)
Teacher	1(3.4)
Lecturer	1(3.4)
Pastor	1(3.4)
Private sector employee	6(20.7)
Self-employed	9(31)
Trader	1(3.4)
Farmer	2(6.9)
Retired	1(3.4)
Student	5(17)
Residence;	
Badung	9(31)
Tabanan	1(3.4)
Gianyar	8(27.6)
Denpasar	9(31)
Buleleng	1(3.4)
Bangli	1(3.4)

The condition of dengue fever which has increased in recent decades along with the emergence of COVID-19, and has caused new problems in the healthcare sector. The incidence of misdiagnosis in the disease will have an impact on the management and prognosis of the patient. This study found 29 cases (1.22%) of misdiagnosis of COVID-19 with dengue fever were found from a total of 2365 COVID-19 cases at Udayana University Hospital from 1 March 2020 to 31 December 2021. The emergence of cases of COVID-19 misdiagnosis with dengue fever is caused by the characteristics of the symptoms and similar laboratory results at the beginning of infection. In addition, it was also reported that the occurrence of false positives through a serological test affected the diagnosis of COVID-19 and dengue fever.[18] There are two possible causes of false positives in the diagnosis using a serological rapid diagnostic

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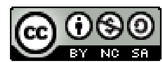


test (RDT). First, patients who experience a false positive have been or are being infected before entering the hospital due to COVID-19. Because of these conditions, it is possible to detect dengue by serological RDT in COVID-19 patients. Second, there was an antibody cross-reaction between COVID-19 and the dengue virus.[15,19,20] Cross-reactions from dengue IgG and IgM are also reported in malaria and leptospirosis. Furthermore, other flaviviruses, such as Zika and Japanese encephalitis, can cause a cross-reaction. The cross-reaction is probably caused by the dengue virus and other flaviviruses that have a large homological structure and sequence. This phenomenon is similar to what occurs in malaria and is thought to be the result of the elicitation of antibody cross-reactions or other immune responses in symptomatic and severe dengue fever to induce infer cross-protection or partial cross-protection.[21] Because of the emergence of a cross-reaction in the use of serological RDT that causes false positive results, the examination of COVID-19 patients and dengue fever patients should use the RT-PCR method to avoid the occurrence of false positive cases.[15,21]

Misdiagnosis cases of dengue fever in COVID-19 are dominated by people over the age of 60. This is most likely due to decreased immunity, which has already begun to decline at that age, making the body susceptible to disease infection. In this study, the majority of cases were found in men with a total of 17 people or 58.6%. Men tend to have higher mobility than women so they have the possibility of being exposed to COVID-19 or higher dengue. This was attributed to greater community contact, including increased outdoor activities, visiting shopping centres, dining in restaurants and bars, and gathering in colleges and universities.[22]

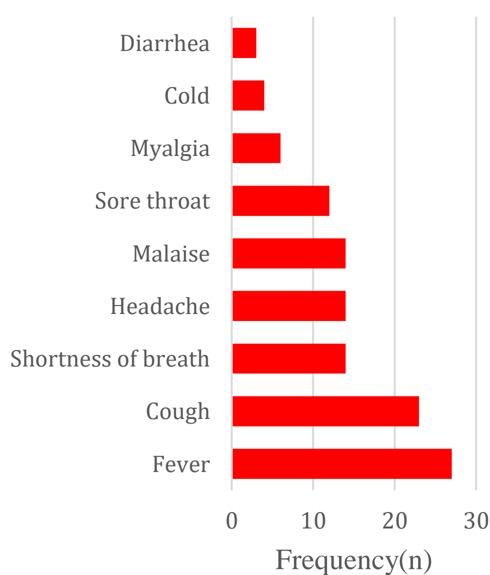
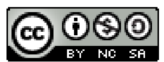


Figure 2. Characteristics of COVID-19 Misdiagnosis Symptoms with Dengue Fever

Based on Figure 2., it was found that the most dominant symptoms experienced by misdiagnosis patients were fever which was experienced by 27 people or 93.1%, followed by coughing which was experienced by 23 people or 79.3%. Shortness of breath, headaches, and sore throats affect 13 people, malaise affects as many as 12 people, myalgia, colds and diarrhea are only found in a few cases, as many as 6 people, 4 people and 3 people in sequence. A systematic review conducted by Tsheten et al. (2021) found similar results, namely that symptoms that arise from cases of misdiagnosis or coinfection with COVID-19 are: fever, shortness of breath, malaise, headache, coughing, rashes, diarrhea, myalgia, nausea or vomiting, and sore throat.[22] Hannan et al. (2022) also mentioned that in their research symptoms that often arise in misdiagnosed patients are fever, myalgia, headache, and diarrhea.[23]



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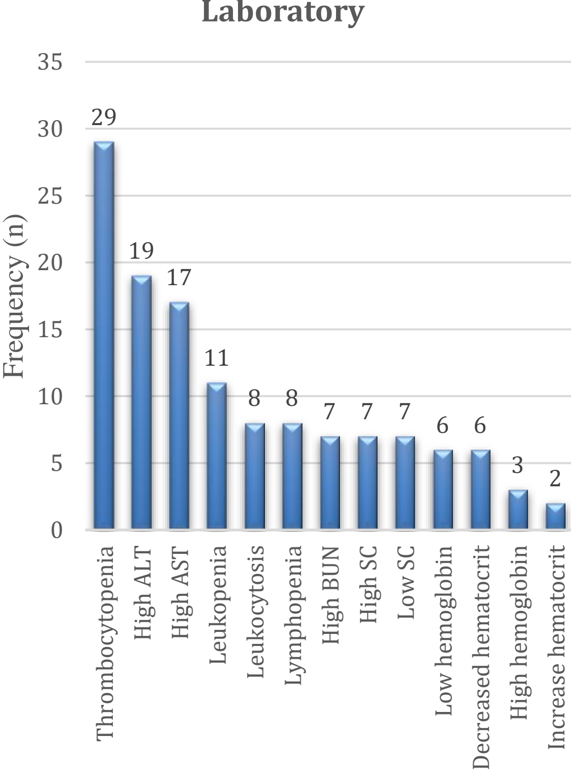

Figure 3. Misdiagnosis COVID-19 Laboratory with Dengue Fever Characteristics

Laboratory characteristics found in misdiagnosed COVID-19 patients troughly serve on Figure 3. include: 29 thrombocytopenia (100%), high ALT in as many as 19 people (65.5%), high AST in 17 people (58.6%), leukopenia in as many as 11 people (37.9%), leukocytosis and lymphopenia in 8 people each (27.6%), high BUN, SC high, and SC low in 7 people each (24.1%), low hemoglobin and hematocrit decreased 6 people each (20.7%), hemoglobin high was 3 people (10.3%), and an increase in hematocrit by 2 people (6.9%). A systematic review conducted by Tsheten et al. (2021) found that in COVID-19, patients with dengue fever had the characteristics of laboratory yields: thrombocytopenia, followed by lymphopenia, high ALT, high AST, high levels of SC, decreased hematocrit, low Hb, leukocytosis, and an increased erythrocyte sedimentation rate (ESR).[22] Meanwhile, Hannan et al. (2022) found laboratory characteristics in COVID-19 diseases and dengue fever: thrombocytopenia, lymphopenia, and hematocrit changes.[23]

The emergence of thrombocytopenia in cases of COVID-19 misdiagnosis with dengue fever is due to decreased platelet synthesis caused by virus induction, which causes bone marrow suppression and platelet clearance. After that, platelets will be destroyed by autoantibodies and immune complexes produced in response to SARS-CoV-2 and dengue virus infections which will cause thrombocytopenia.[22] Leukopenia is characterized by severe thrombocytopenia with an increase in hematocrit due to plasma leakage.[24] Leukopenia and leukocytosis can occur together with lymphopenia as an indicator of the severity of the disease. Leukopenia and lymphopenia can be used as markers to distinguish infections.[25] The occurrence of lymphopenia is caused by dengue virus infection of hematopoietic progenitor cells, dengue T cell activation, and marrow stromal cell infection. This results in the release of cytokines that cause lymphopenia.[24]

Aminotransferase (ALT and AST) is an enzyme used as a marker of hepatocellular damage. In dengue fever, increased aminotransferase becomes a sign of the severity of the disease due to the dengue virus will make the liver a target of infectious organs. Meanwhile, in COVID-19 aminotransferase levels are generally normal or experience a slight increase.[26]

Table 2. Conditions and length of stay of COVID-19 misdiagnosis patients with dengue fever.

Conditions	n (%)
Recovered	24(82.8)
Dead	5(17.2)
Length of stay	4.38 days

Based on Table 2. The length of stay the patient has been hospitalized has an average of 4.38 days. The patients included 24 people (82.8%) who healed and 5 people (17.2%)

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who died. The occurrence of misdiagnosis of COVID-19 and dengue fever will result in mistakes in disease management. This affects mortality and morbidity, worsening the patient's prognosis.[22]

STRENGTH AND LIMITATION

The strength of this study was that it is the first literature to discuss the co-infection of COVID-19 with dengue fever so that it can be used as a guide and literacy material for future research. With this research, it is expected to be a benchmark in diagnosing COVID-19 and dengue fever so that there are no errors in the diagnosis of the disease.

The limitation of this study was that it was conducted using a cross-sectional research design with a small sample and a limited period time. It is expected that in the future research can be carried out with a larger sample and a longer period time so that the results of the study can represent the general population. The study was carried out in a single hospital, so it described only a limited population. Data collection for the study is carried out using secondary medical records, so further validation is needed for data acquisition. In addition, the diagnosis of dengue fever doesn't use virus isolation and nucleic acid detection techniques such as RT-PCR. In this study, the diagnosis of dengue fever was established based on clinical symptoms, laboratory results, and serological tests such as IgG, IgM, and NS1, thus allowing for false positives in making the diagnosis.

CONCLUSIONS

The incidence of misdiagnosis of COVID-19 with dengue fever at Udayana University Hospital in 2020-2021 was 29 people (1.22%) of a total of 2365 COVID-19 cases. The most common symptoms complained of by patients are fever, followed by coughing, shortness of breath, headache, and sore throat. The laboratory results obtained in this study were thrombocytopenia, followed by lymphopenia, high ALT, high AST, high BUN, high SC, low SC, decreased hematocrit, low Hb, high Hb, and increase in hematocrit. Misdiagnosis COVID-19 with dengue fever must receive special attention. The similarity of the symptoms and laboratory results of the two diseases allows for a diagnosis error that will affect the patient's management.

AUTHOR CONTRIBUTION

Writer, literature searcher, collecting data from literature: IKHA, Conceptor and supervision: IKS, review and supervision: IKS and NLPED. Both IMS and CAWP contributed to the review and editing of the final version of the manuscript and Project administration of the manuscript.

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CONFLICT OF INTEREST

There was no conflict of interest in making this scientific work.

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Original Article

An Initiative Report on Hospitalized Pulmonary TB Patients Co-Infected by SARS-CoV-2 during the COVID-19 Pandemic from Tertiary Referral Hospitals in Surabaya

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ABSTRACT

The enduring effect of SARS-CoV-2 pandemic has been experienced throughout the past and ongoing three years. Incidences of SARS-CoV-2 co-infected tuberculosis patients were reported globally, including in Italy and several European countries and resulted in a more complicated disease with severe clinical features and poorer clinical outcomes. To effectively manage this co-infection, it is important to be informed of the prevalence and characteristics of an acute SARS-CoV-2 co-infection on TB and determining factors of severity. Therefore, early warning signs can be recognized, monitored closely and managed. This retrospective study, carried out on hospitalized TB patients in Dr. Soetomo Hospital and Universitas Airlangga Hospital, Surabaya, Indonesia, used medical records from March 2020 to December 2022. Samples were from inpatients with a molecularly-Gene Xpert MTB/Rif-confirmed tuberculosis, and currently experienced respiratory and fever symptoms that resembles the symptoms of SARS-CoV-2 infection or exacerbation of tuberculosis. They are then screened and examined using a molecular diagnostic test, with real-time RT-PCR for SARS-CoV-2. A total of 54 (0.7%) patients had TB-SARS-CoV-2 co-infection among 7,786 suspected to have TB, of which 35 had Rifampicin Sensitive (TB-RS), while 19 had TB Rifampicin Resistant (TB-RR) co-infected with SARS-CoV-2. The remaining 2,586 suspected TB patients had only MTB, based on the detection methods of X-pert MTB/RIF, but with negative RT-PCR of SARS-CoV-2. The clinical severity and mortality of TB-SARS-CoV2 co-infected patients were significantly associated with the number of co-morbidities (p=0.0156), and serum haemoglobin levels (p=0.0672), in which p value < 0.05 is considered significant.

Keywords: TB-SARS-CoV-2 co-infection, clinical severity, Sensitive Rifampicin, Resistant Rifampicin, Tuberculosis

Highlights: The pandemic of SARS-CoV-2 is associated with incidence of SARS-CoV-2 co-infection in tuberculosis patients, leading to a more complex disease activity with severe clinical features. This research aims to strategically enhance services for the management and prevention of SARS-CoV-2 and tuberculosis co-infection.

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INTRODUCTION

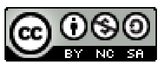
The first reported case of novel coronavirus in Wuhan in late 2019 has led to the World Health Organization declaration of global pandemic Corona Virus Disease of 2019 (COVID-19). This existing pandemic has placed a significant concern of Case Fatality Rate as high as 16.7%. This concern is especially felt for those who are more vulnerable and have comorbidities such as elderly and those with underlying lung disease as in Tuberculosis. [1,2]

There have been documented cases of co-infection between Tuberculosis and Severe Acute Respiratory Syndrome Coronavirus 2 (TB-SARS-CoV-2), which occurred globally such as reported by Stochino et.al, 2020 and Tandolini et.al, 2020.[3,4] This previous studies suggested that SARS-CoV-2 infection can occur independently of TB, either before or during, or after the disease[5,6]. However, this study defined co-infection as an endemic underlying Tuberculosis which are then exposed and co-infected with SARS-CoV-2. The tuberculosis disease state with weakened immunity is therefore more susceptible to contracting emerging viral SARS-CoV-2 respiratory disease. This co-infection might further affect the drug resistant TB problem.[7-10]

This study aims to describe the prevalence and characteristics of an acute SARS-CoV-2 co-infection on chronic TB, which primarily affects the lungs. in our region, and determining factors of susceptibility, severity and prognosis. Previous study reported that ages such as the elderly, the highly susceptible host experienced a rapidly fatal illness from a single SARS-CoV-2 infection with the generalized spread of the disease to many organs[3,4]. Meanwhile, at younger ages, which considered more resistant hosts, it can cause a mild COVID respiratory syndrome. Therefore, it could be assumed that a more severe disease may occurred in elderly hosts with weakened immune status due to previous TB infection. An understanding of the vulnerable TB host factor and the pathogenesis of TB-SARS COV2, which is needed to prevent the incidence of co-infection as well as to diminish the severity and fatality. It is also expected to help in compiling a reliable and validated human and environmental health protocol.

Stochino et.al, 2020 and Tandolini et.al, 2020 identified the risk factors for severe coinfection of TB-SARS-CoV-2, namely endogenous (host), exogenous (agent of infection), and environmental factors[8,9]. The endogenous components include high-risk age groups (elderly), genetic factors, nutritional status (malnutrition both underweight and overweight), as well as comorbidities, such as underlying TB-endemicity, TB chronicity, and other respiratory diseases. They also consist of immunosuppression conditions, including diabetes, renal function disorder, as well as underlying chronic and progressive viral infections, such as HIV, Hepatitis B, and Hepatitis C. The exogenous components that increase the power of infectious agents' transmission include the similarity of SARS-CoV-2 and MTB transmission through close contacts, airborne, and droplets. They also have a similar mechanism of evading the host immune system by replicating intracellularly within host macrophages and epithelial cells. Consequently, complete viral and bacterial clearance is difficult to achieve. The level of education and awareness in the community is still lacking. There is also a prevalence of ignorance and hesitancy in the community as well as poor environmental health. These components increase the rate of transmission, and they cause higher severity clinical outcomes and mortality in TB co-infected SARS-CoV2 patients[8,9].

Several studies on the co-infection of TB-SARS-CoV-2 showed that TB can significantly reduce SARS-CoV-2 specific response, and it is characterized by low



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lymphocyte count[10]. This reduced or absent response to SARS-CoV-2 antigens is caused by massive compartmentalization of the specific T-cells in infectious foci, or by the elimination of effector T-cells when fighting high doses of antigens[11]. The co-morbidity TB-COVID-19 does not have a direct impact on SARS-COV2-specific response, and it is associated with worse clinical outcomes[9]. There is a dual critical impact where COVID-19 pandemic worsened TB epidemic globally due to TB-services fragmentation and the additional pressures on health systems, which weakened the National TB programs [12–15]. RT-PCR (Real time PCR) is a simple, reliable, and rapid test that is widely used for the detection of patients with TB and without TB coinfected with SARS-CoV-2[16–21]. It has a technical limit of detection (LOD) < 10 copies/ reaction and a detection threshold of 3.8 RNA molecules per reaction[22]. These parameters depend on the amplified region as well as the primers and probes used in the RT-PCR platform analysis. Therefore, this study particularly aims to describe the demographic profiles and their clinical characteristics in hospitalized TB patients coinfected with SARS-CoV-2 in two tertiary referral hospitals in Surabaya, followed by an analysis of its correlation with the clinical severity of TB-SARS CoV-2 co-infection.

MATERIAL AND METHODS

Ethics statement

This study was approved by the RSDS ethics committee (Ref. No. 0492/LOE/301.4.2/VI/2021) and RSUA ethics committee (Ref. No. 185/KEP/2021). The data used were collected from documented records and laboratory reports.

Materials

This is a retrospective study, where information and data were collected from medical and laboratory records of hospitalized TB patients in two tertiary referral hospitals, namely Dr. Soetomo Hospital and Universitas Airlangga Hospital, Surabaya, Indonesia during the COVID-19 pandemic between March 2020 and December 2022.

Methods

Incoming patients aged > 18 years who were suspected to have TB were included as participants, and the prediction was confirmed using Xpert MTB/RIF[23]. Children aged < 18 years were excluded in this retrospective study because of the nonspecific clinical and radiologic sign of TB and tend to present in paucibacillary disease. The patients who met the inclusion criteria were then grouped as TB Rifampicin Sensitive and TB Rifampicin Resistant. Subsequently, the confirmed TB inpatients were tested for SARS-CoV-2 coinfection using real-time RT-PCR[12]

The cases were retrospectively recorded using a logbook, including demographics, evidence of SARS-CoV-2 infection, clinical characteristics, co-morbidities, disease course, laboratory, imaging, and recovery/outcomes. Classification of weight was categorized using BMI measurement in adult Indonesian, as described in the Table 1.1.

Table 1.1 Classification of weight by BMI in adult Indonesia

Classification	BMI (kg/m^2)
Underweight	< 18.5
Normal range	18.5-22.9
Overweight	≥ 23
At risk	23-24.9
Obese I	25-29.9
Obese II	≥ 30

The patients were used for analysis if they are positive for TB using the gene Xpert molecular testing and confirmed with SARS-CoV-2 infection based on WHO criteria, namely a positive PCR[12,21,24]. The severity of TB was then assessed with the Modified

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Bandim Score, while that of COVID-19 was evaluated using NIH criteria [23,25,26].

Statistical Analysis

The positivity RT-PCR and clinical characterization data were recorded and further described in the distribution table. A statistical correlation test was used to analyse collected data. The cut off p-value of significance is p<0.05.

RESULTS AND DISCUSSION

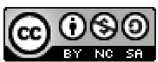
Between March 2020 and December 2022, 54 (0,7 %) TB co-infection SARS-CoV-2 cases were found among the 7,786 who were examined with the GeneXpert MTB/RIF and RT-PCR SARS-CoV-2. Among these 54 patients, 35 were diagnosed with SR-TB, while 19 had RR-TB, both of which co-infected with SARS-CoV-2.

From Table 1.2, it can be seen that 54 confirmed cases of TB coinfected with SARS-CoV-2 patients, 11 (20%) died (14% from SR-TB, 32% from RR-TB). There were 44 recovered from SARS CoV-2 coinfection. From Table 1.3, mean hemoglobin for coinfected patients was 8.5g/dL for TB-RIF sensitive-mild COVID-19, 10.7g/dL for TB-RIF sensitive-moderate COVID-19, 9.16g/dL for TB-RIF sensitive-severe COVID-19, 11.25g/dL for TB-RIF resistant-mild COVID-19, 11.05g/dL for TB-RIF resistant-moderate COVID-19, 11.1g/dL for TB-RIF resistant-severe covid, for deceased case 8.3g/dL , for recovered case 10.68g/dL. There was a nearly statistically significant association (p=0.06) between the concentration of hemoglobin in the blood and mortality. The mortality rate was found to be higher in individuals with lower hemoglobin levels.

Most 40% confirmed TB-RIF sensitive co-infected with COVID patients had two co-morbid, 37% had one co-morbid, 16% had three co-morbid, 7% had four co-morbid, whereas most 54% confirmed TB-RIF resistant coinfected with COVID patients had three co-morbid, 18% had four co-morbid, 9% had one co-morbid, 18% had two co-morbid. There was a statistically significant association (p=0.01) between number of co-morbid and mortality in which, there was higher mortality rate with the increasing number of co-morbid disease. The most frequent co-morbidities were anemia (39%) and diabetes mellitus type 2 (30%). Compared with survivors, deceased cases showed a higher prevalence of co-morbidities within TB-RIF resistant including anemia(27% vs. 4%), diabetes (27% vs. 9%). Dyspnea (56%), cough (69%), and fever (26%) were the most frequent clinical symptoms.

Table 1.2 Characteristic individuals TB-RS and TB-RR coinfected with SARS-CoV-2

TB Categories	**TB Rifampicin Sensitive (N=35)**			**TB Rifampicin Resistant (N=19)**		
COVID Severity	Mild (N=6)	Moderate (N=22)	Severe(N=7)	Mild (N=2)	Moderate (N=12)	Severe (N=5)
Age (years)	42(21-68)	49(20-69)	44(22-62)	42(29-54)	41(23-61)	46(21-61)
Gender						
Female	3 (50%)	9(41%)	4(57%)	2(100%)	5(42%)	3(60%)
Male	3(50%)	13(59%)	3(43%)	0	7(58%)	2(40%)
Nutritional status (BMI in kg/m²)						
Normal	3(50%)	15 (68%)	1(14%)	2(100%)	8(67%)	3(60%)
Underweight	2(33%)	5(23%)	2(29%)	0	4(33%)	2(40%)
Overweight	1(17%)	2(9%)	4(57%)	0	0	0



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Outcome						
Survive	5(83%)	21(95%)	4(57%)	0	10(83%)	3(60%)
Nonsurvive	1(17%)	1(5%)	3(43%)	2(100%)	2(17%)	2(40%)
>1 co-morbid	5(83%)	14(64%)	6(86%)	0	6(50%)	5(100%)
Anemia	5 (83%)	8(36%)	1(14%)	0	2(17%)	3(60%)
DM type 2	2(33%)	6(27%)	3(43%)	0	4(33%)	3(60%)
Hypertension	0	1(5%)	1(14%)	0	1(8%)	1(20%)
Hyperthyroid	0	0	0	0	0	1(20%)
HIV	1(17%)	1(5%)	0	0	0	1(20%)
Hep B	0	0	1(14%)	0	1(8%)	1(20%)
Hep C	0	0	0	0	1(8%)	0
Acute Kidney Failure	0	2(9%)	1(5%)	0	0	0
Chronic Kidney Failure	0	1(17%)	0	0	0	1(20%)

Table 1.3 Comparison of mean laboratory values among TB-RS and TB-RR coinfected with SARS-CoV-2.

TB categories	**TB Rifampicin Sensitive (N=35)**			**TB Rifampicin Resistant (N=19)**		
COVID-19 severity	Mild (N=6)	Moderate (N=22)	Severe(N=7)	Mild (N=2)	Moderate (N=12)	Severe (N=5)
Laboratory results						
WBC (µL)	7,947	10,735	11,911	5,960	7,550	9,394
NLR	7.88	13.15	19.92	4.31	5.56	8.19
Monocyte (µL)	826	797	833	585	716	834
CRP (µg/mL)	7.02	24.53	13.51	2.62	2.8	3.325
Length of positive CT value (days)	25.5	15.5	12.29	15	29	9.4

Table 2. Correlation Analysis of Several Determining Factors and Mortality Outcome

Determining Factors	***p values, significant if p < 0.05***
Age	p = 0.6106
Gender	p = 0.6418
Nutritional status	p = 0.1092
Co-morbidity	**p = 0.0156**
Haemoglobin	**p = 0.0672**
WBC	p=0.5537
NLR	p=0.2201
Monocyte	p=0.2283
CRP	p=0.1088
TB category	p=0.1369
COVID severity	p=0.4580

In this study has been shown in Table 2., the two most common co-morbid diseases were anemia and type 2 diabetes mellitus. The most common co-morbidity in TB-SARS-CoV-2 co-infection, being present in about 41% of coinfected patients, was anemia. This association, which seems to be more frequent in women, was directly influenced by aging and concomitant presence of CKD. More importantly, TB-SARS-CoV-2 co-infected patients with anemia had an approximately higher risk of death (p=0,0672) in the short-term period compared to those without. Our findings are in accordance with the results presented by Al-Jarallah et al. (2021) who reported that COVID-19 patients having a hemoglobin > 10 g/dL had lower odds of dying than those who were considered anemic (i.e., Hb < 10 g/dL). From a pathophysiological perspective, Hb concentration represents one of the most important markers of oxygen-carrying capacity in the bloodstream. Therefore, anemia can further reduce oxygen delivery to

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peripheral tissue in COVID-19 patients who have an increased oxygen demand due the interstitial pneumonia [7,23,24,35]. Another major contributing role could have been played by the impairment of iron metabolism due to the underlying infection, resulting in the reduced availability of the metal for erythropoiesis and the production of Hb.[8,35] Whereas, diabetes mellitus type 2 has a typical adult-onset of insulin resistance, and manifest as increased blood glucose level. If the elevation in blood glucose level is uncontrolled, it can lead to a decrease in neutrophil function, response from T cell lymphocyte, antioxidant status function, and altered secretion of proinflammatory cytokines. This defect in modalities of systemic immune functions increases the virulence of opportunistic pathogens. In addition, the condition of high blood sugar also provides nutrition for microbes, thereby further increasing the virulence of microbial infection, including opportunistic bacterial and candida infections.[27,28] Whereas patient with co-morbidities of other viral infection, such as HIV, Hepatitis B, Hepatitis C, are prone to be underweight, carbohydrate-fat and protein deficiency, depleted lymphocyte and T-cell counts, decreased immunomodulatory effects and so might contribute to exacerbation of coinfection manifestation thus, increases the risk of mortality. Another study revealed that the presence of impaired nutritional status increased the risk of an abnormal and chronic inflammation with higher level of oxidant. Failure to eliminate agents of coinfection can aggravate clinical outcomes of TB-RS and TB-RR with SARS-CoV-2. The presence of Rifampicin resistant TB can cause an obscure clinical manifestation of coinfection exacerbation. This is due to failure in eradicating rifampicin resistance tuberculosis bacilli and it is more difficult to activate optimal immune response to an acute SARS-CoV2 infection agent in Tuberculosis infected macrophage [11,17,19,32–34].

Pathophysiology of co-infection TB-SARS-CoV-2 depends on the number of initial viral load, ability to evade from the host immune system by replicating intracellularly within macrophages, and the severity of underlying chronic TB infection. It can also be influenced by the presence of endogenous risk factors, inflammatory responses of the host innate, and adaptive immune system in eliminating coinfection effectively. Severity of clinical outcome is often affected by hyperactivity of the host immune response, and it is characterized by the production of cytokine storms, which cause systemic organ hyperinflammation and destruction.

Consequently, there was no significant difference in the time length of positivity for the E, N, ORF1ab gene of SARS-CoV-2 in TB-RR group lasted within 9-15 days was needed to clear SARS-Cov-2. In cases of new TB patient with Rifampicin Sensitivity, 12-25 days were required. Early detection of TB-SARS-CoV-2 coinfection using E, N, ORF1ab gene, as well as RdRp and Helicase gene gave the same accuracy in indicating active replication and ongoing pathogenicity[31].

This study highlights the importance of concomitant molecular detection pathway for TB patients experienced exacerbation of symptoms during their antituberculosis medication regimen, using Gene Xpert MTB/RIF and RT-PCR SARS-CoV-2 for effective both SR/RR-TB-SARS-CoV-2 co-infection case findings and then delivering early treatments. It can also help to prevent further or break the transmission chain of SR-TB-SARS-CoV-2 co-infection and RR-TB-SARS-CoV-2 co-infection.

STRENGTH AND LIMITATION

The strength of this study was the first preliminary study carried out from two referral hospitals in Surabaya that reported incidences of TB-SARS-CoV-2 coinfection based on detection of SARS-CoV-2 gene using molecular RT-PCR methods in diagnosed and hospitalized TB patients. This

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study has limitations. The reporting system relies on consecutive sampling which included a limited 54 TB patients with SARS-CoV-2 coinfection from 7,786 suspected TB patients collected during the peak exponential COVID-19 pandemic. Therefore, the result of this research study, needed to be confirmed with larger samples of clinical study.

CONCLUSIONS

Small incidences of 54 cases of TB-SARS-CoV-2 co-infection was found in two tertiary referral hospitals in Surabaya using molecular RT-PCR assays. There was no significance difference in profile prevalence of gene specific SARS-CoV-2 detection or CT value between TB-RR and TB-RS groups. As all confirmatory gene specific SARS-CoV-2 are detected, in both methods using E, N, ORF1ab gene as well as methods using RdRp and Helicase gene detection. There was a significant difference between 35 patients of SR-TB and 19 patients of RR-TB in terms of their clinical severity and mortality outcomes. The clinical severity level and mortality of TB-SARS-CoV-2 co-infected patients were significantly associated with the number of co-morbidities (p=0.0156) and serum hemoglobin concentration (p=0.0672). Thus these comorbidities and the level of serum hemoglobin need to be considered as warning signs, monitored closely and managed.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

LE, NMM, SS, PDE have equally contributed to the designing, data analysis, interpretation of data, drafting or revision of critically important intellectual content, given final approval of the version to be published.

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Original Article

Polyvinyl Chloride (PVC)-Glycerol with Chitosan Addition for Antibacterial Blood Bag Application

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ABSTRACT

Blood bag is a medical device that stokes and transports whole blood or blood components. The material that is often used for blood bag membranes is Polyvinyl Chloride (PVC), however the common problem that is bacterial contamination and that material have not antibacterial characteristic. Because of this matter, the aim of this reseach are a blood bag that has antibacterial function is needed and meet the ideal standard as bloodbag. Chitosan as a blood bag membrane material fabrication to get the antibacterial effect. Chitosan is chosen as a blood bag material fabrication to get the antibacterial effect. Chitosan has several specific biocompatibility properties, antibacterial, chelation, and biodegradability. This study used various Chitosan concentrations of 1.5%, 2%, 2.5%, and 3%, and Glycerol was added as a plasticizer. The composition of Chitosan: Glycerol is 1:1. Then, the mixture is added to the PVC solution in a ratio of 1:5 then poured into a petri dish. The results showed characterization that the biocomposite PVC-Glycerol with the addition of 3% concentration of chitosan was the best composition, the tensile strength test result of biocomposite is 21.20 MPa, the absence of membrane pores in the morphology of the blood bag, the hemolytic activity is 0.24%, and the inhibition zones of *E. coli* and *S. aureus*, respectively 11.66 mm and 12.66 mm in diameter. Based on the characterization results, the biocomposite PVC-Glycerol membrane with the addition of Chitosan has a very high potential as a candidate for blood bag membranes.

Keywords: Antibacterial, Blood bag, Membrane, Plasticizer, Poly Vinyl Chloride

Highlights: Bacterial contamination occurs during the process of taking and processing blood that is less aseptic because the issue material blood bag must have antibacterial. The PVC-glycerol-chitosan composites can be good candidates for ideal blood bag membranes because meet the standards of mechanical, physical, and biological tests.

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INTRODUCTION

Currently, bacterial contamination of blood products is still a serious problem because it has a risk of fatal transfusion with the risk of bacterial sepsis.[1] Many cases of bacterial contamination still occur because of the Thrombocyte Concentrate (TC) storage suitable for bacterial growth. The source of bacterial contamination is obtained from the process of taking and processing blood that is less aseptic. Bacterial contamination is still a serious problem in the world with the risk of bacterial sepsis.[2,3] It also becomes an important problem in Indonesia due to the limitations of bacterial detection tools almost in every blood donation unit (UDD). The source of contamination bacteria can come from donor skin less aseptic, donor bacteremia, and processing of blood products.[3,4] Besides that, the storage conditions of Thrombocyte Concentrate at 20-24°C, processing at porous bag with agitation process, as well as the addition of preservatives in TC storage pouch can be an energy source for bacteria growth of contaminant bacteria getting better.[5] Cases of bacterial contamination have a risk of infection through higher blood transfusion than viral infection. Studies previously showed that 9.2% of 196 blood products are known to be contaminated with Gram bacteria positive and Gram-negative bacteria.[6] Results identification shows that there are staphylococci, *Bacillus sp., Pseudomonas, Streptococcus pneumoniae, and Pseudomonas aeruginosa* in products stored in the blood. More than 50% of bacteria detected in the product TC blood are Gram-positive bacteria that can cause a transfusion reaction while Gram bacterial contamination negative is usually less however in the case of Gram bacterial contamination negative has a transfusion risk of up to one death.[6,7]

Blood is a very important part of the human body. The main function of blood in the body is to transport oxygen and substances needed in the body. Blood deficiency in humans causes several diseases such as anemia, thalassemia, leukemia, sepsis, hemophilia, and kidney failure.[8] The main treatment of blood deficiency disease is the administration of blood transfusions by maintaining a hemoglobin level above 10 g/ml.[9] Blood transfusion is the infusion of blood components from one individual (donor) to another individual (recipient). Blood transfusion requirements must have a match between donor red blood cell antigens and plasma antibodies or recipient serum so that no hemolytic reactions occur.[10] The high need for blood transfusion is directly proportional to the amount of blood bag needed. The average need for blood in the hospital reaches 100 bags of blood per day with a blood size of 350 cc.

Under normal conditions, the amount of blood supply needed is 300 bags[11], from the number of blood bags found defects in several bags of blood. Blood bag defects in the form of contamination of the blood bag by its ingredients, so that the blood in it becomes improper to transfuse. In addition, the Indonesian Red Cross (PMI) in several cities for several years destroyed around 1,000 blood bags that were not suitable for use. Besides its reactive nature and blood from the donor is contaminated with bacteria. Bacterial contamination was observed in 18 (9.2%) of the blood and blood components, of which 14 (77.8%) and 4 (22.2%) were Gram-positive and Gram-negative bacteria, respectively. The physical properties of blood bag material and bacteria are the main factors that cannot be used for blood vessels. The blood bag must be transparent, not damaged when bent on a small radius, flexible, heat resistant during the sterilization process, not easily damaged during the centrifuge, economical, and handling.[12] Many blood bags on the market today are made of Polyvinyl Chloride (PVC) with a plasticizer mixture. However, there is much evidence that blood

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bags are exposed to or contaminated with bacteria, so there is a need for antibacterial blood bags.[13] The antibacterial property of blood bags aims to minimize the spread of bacteria in blood so the diseases caused by bacteria in the blood can be reduced. Antibacterial effects found in blood vessels are expected to be integrated with Polyvinyl Chloride (PVC)-glycerol biocomposite so that the original nature of the blood bag formed is maintained. With the presence of an antibacterial blood bag, blood damage due to bacterial contamination in the blood can be reduced.

One of the natural ingredients containing antibacterial is chitosan biopolymer. Chitosan is a chemical compound derived from chitin.[14] The addition of chitosan to Polyvinyl Chloride (PVC)-glycerol is intended to have antibacterial properties in the blood bag, in addition, chitosan biopolymers have properties that are bioactive, biocompatible, hemostatic, and can be biodegradable.[13,15] This study will modify the blood bag made from PVC-glycerol by adding chitosan to increase the antibacterial properties of blood bags. Research on PVC-glycerol-chitosan composites has been carried out by several previous researchers[12] who synthesized the composite with a ratio of chitosan-glycerol 1:1 and varied the concentration of chitosan between (0.5–2) wt/v%. The study results showed the presence of antibacterial properties on the membrane. In addition, the increase in chitosan concentration was also followed by an increase in tensile strength, but its value did not meet the standard tensile strength used as a bag of blood. The research of Omer et al[13] The PVC with the addition of clove oil showed antibacterial activities against four different bacterial strains (two-Gram positive: *Staphylococcus aureus* and *Bacillus cereus* & two-Gram negative: *Pseudomonas aeruginosa* and *Escherichia coli*.

The addition of chitosan is to provide antimicrobial and good biocompatibility.[16] Chitosan showed an intrinsic antibacterial activity, impeding bacteria and fungi growth.

As an example, in Staphylococcus aureus cultures, chitosan stimulate structural changes membrane–the wall complex leading to the impairment of surface cell structures and bacterial death.[17] The standard tensile strength in a blood bag using PVC material is 14-26 MPa.[18] This research will focus on increasing tensile strength and antibacterial values by increasing the concentration of chitosan on PVC-glycerol-chitosan composites because it can increase the tensile strength of a blood bag. Thus the purpose of this study is to obtain blood bags that have physical, mechanical, and biological properties in accordance with the blood bag standard. Based on empirical research data[6] it is predicted that the greater the concentration of chitosan in PVC-glycerol-chitosan composites will increase the antibacterial properties. In addition, it is predicted that it will also increase tensile strength. Microscopic and macroscopic observations to determine the behavior alteration of the composite material. Microscopic observation of blood bag material was carried out through the Fourier Transformed Infra-Red (FTIR) and Scanning Electron Microscopy (SEM) tests. The macroscopic observation will be carried out through a tensile test, while the biological test will be carried out through an anti-bacterial test. In addition to these tests, a hemolysis test was also carried out to determine the interaction of blood with blood bag material, especially the response of red blood cells to the material.

The aims of this study are: 1) To explore the effect of chitosan addition on the PVC-glycerol on tensile strength, functional cluster, superficial surface morphology and pore size, anti-bacterial ability, and blood bag hemolysis percentage. 2) To know the optimal concentration of chitosan for PVC- chitosan-glycerol blood bag.

MATERIAL AND METHODS

Materials

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Control variables in this study Polyvinyl Chloride (PVC) Concentration The independent variable of this study is the addition of Chitosan concentration 0 wt/v%, 1.5 wt/v%, 2 wt/v%, 2.5 wt/v% and 3 wt/v%, and the dependent variable is the characteristics of PVC - Biocomposite glycerol - chitosan. The materials used in this study were Polyvinyl Chloride (PVC), Chitosan (0%, 1.0%, 2.0%, 2.5%, 3.0%), Glycerol, Acetic Acid, Tetrahydrofuran (THF), Aquades. The tools used are a digital balance sheet, glass beaker, micropipette, petri dish, measuring cup, spatula, weigh paper, and magnetic stirrer. The tool used to carry out the characterization is Tool 8400 Shimadzu FTIR for the FTIR test, Imada HV-500 NII Autograph to determine tensile strength, water bath (Gemmyco YCW) for hemolysis test, petri dish for the antibacterial test, and using spectrophotometric UV-VIS device (Shimadzu UV-1800).

Methods

Blood Bag Synthesis Procedures

The blood bag is made by mixing 10% PVC dissolved tetrahydrofuran (THF) using a magnetic stirrer. Antibacterial blood bags made from chitosan dissolved in 1% acetic acid five variations of the solution were made with different concentrations of chitosan. Blood bag membrane with chitosan concentration of 0 wt/v%, 1.5 wt/v%, 2 wt/v%, 2.5 wt/v%, and 3 wt/v% and 10% PVC with a ratio of 2:10 and solution glycerol with chitosan comparison 1: 1. The mixing process uses a magnetic stirrer.

Functional Group Test

This test is used to analyze functional groups of organic and inorganic compounds. The test was performed using a tool called Fourier Transform Infrared (FTIR) Shimadzu, 8400S.

The samples identified can be either solid samples or samples. The peak value of the light that didn't get absorbed by the detector is then processed using a computer using the Fourier transform method that could be calculated using the following formula:

$$F(\omega) = \int_{-\infty}^{\infty} f(t)e^{(-j\omega t)}dt \qquad (1)$$

With f(t) as the original signal in the time domain, it will be transformed into F(ω), a function in the frequency domain by a continuous integral function. From this transformation, it will produce a graph representing the chemical bonds in the compounds contained in the sample.[19]

Morphology Test

The Morphology Test was carried out using Zeiss's scanning electron microscope (SEM). Samples were cut transversely, sputtered with gold-palladium, then observed under SEM.

Hemolysis Test

The hemolysis test was performed using human blood that had been given an anti-coagulant, *Ethylene Diamine Tetraacetic Acid Dipotassium Salt (EDTA).* Blood-EDTA mixture taken 200 Ul was diluted using 10 Ml of saline with a concentration of 0.9% then inserted into a micro tube, each tube containing 200 ul as a negative control. Blood-EDTA 200 ul was diluted with 10 ml of distilled water, after which 200ul was taken as a positive control. The sample was inserted into a microtube containing blood with saline and then incubated for two hours using a water bath at normal temperatur (37°C).

Antibacterial Test

The antibacterial test aims to determine the ability of the PVC-Glycerol-Chitosan biocomposite membrane and mangrove extract to inhibit bacterial growth.

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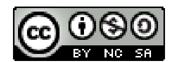

This test was carried out using two bacteria, namely *S.aureus* bacteria representing gram-positive bacteria and *E.coli* representing gram-negative bacteria. The strength of antibacterial inhibition was classified as weak showing an inhibition zone of ,<5mm, said to be moderate showing an inhibition zone of 5-10 mm, said to be strong showing an inhibition zone of 10-20 mm and said to be very strong when showing an inhibition zone of more than 20 mm[12].

Tensile Test

The tensile test is a destructive engineering and materials science test whereby controlled tension is applied to a sample until it fully fails. This is one of the most common mechanical testing techniques. It is used to determine how strong a material is and how much it can be stretched before it breaks.

The variable of the tensile test is carried out using a Shimadzu AGS-X tool using a tensile test frame of paper, with a gauge length of 10 mm. The sample is attached to the tool, then the frame is cut. Samples are drawn at a speed of 5 mm/minute (ASTM D 882–02).

RESULTS AND DISCUSSION

Fourier Transform Infrared (FTIR)

Characteristics of functional groups from PVC-glycerol biocomposite membrane samples with chitosan addition were analyzed using Fourier Transform Infrared (FTIR). The results of the FTIR spectrum of PVC-Glycerol biocomposite membrane samples with the addition of chitosan are shown in Figure 1.

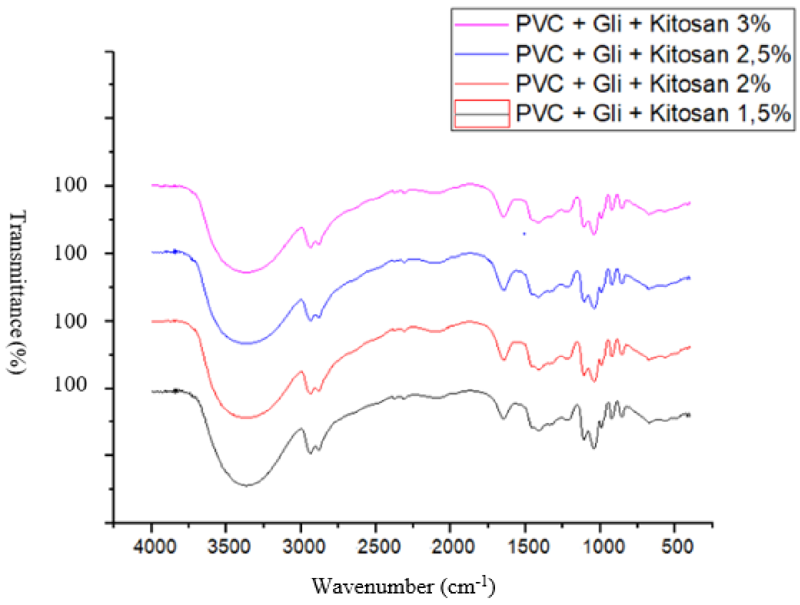

Figure 1. Results of the FTIR Test

On the results of the FTIR characterization of PVC material, it is known that there is a C–H strain with a wave number of around 2970 cm^{-1}. Typical absorption on PVC material appears at a wave number of 1425 cm^{-1} which is the CH_2 functional group. The C–H trans functional group is found at a wave number of around 960 cm-1 and there is a C-Cl stretch with an absorption wave number of 682 cm^{-1} on PVC material.[13] The FTIR spectrum of glycerol material has a wave number of around 3390 cm^{-1} indicating the presence of the –OH functional group. The absorption at wave number 2939 cm^{-1} indicates the C-H functional group. the CH_2 functional group is at the peak of wavenumber 1416 cm^{-1}while the C-O functional group is also visible at the absorption wave number 1110-1043 cm^{-1}.[12]

Test results In the FTIR spectrum of chitosan, there is an absorption at a wave

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number of 3433 cm^{-1}, indicating a stretch of the -OH functional group. Chitosan material has a typical absorption seen at the absorption wave number of 1647 cm^{-1} indicating the N-H functional group of the amine (NH_2) and the absorption wave number of around 1151 cm^{-1} indicating the C-N functional group [20]. The FTIR test results for the PVC-Glycerol biocomposite membrane show the presence of an -OH group at a wave number of 3381 cm^{-1}, a CH_2 functional group at a wave number of 1423 cm^{-1}, a C-H functional group at a wave number of 2939 cm^{-1} and a C-O functional group at a wave number of 2939 cm^{-1}. Wave number 1111-1043 cm^{-1} contained in the glycerol material. The stretch function group C–Cl with a peak wave number of 675 cm^{-1}, and the trans C–H functional group at a wave number of around 960 cm^{-1} comes from PVC material. Membranes that have been tested FTIR showed absorption wave numbers that indicate the functional groups of PVC material, glycerol, and chitosan. In PVC material, it is known that there is a peak at the wave number of 2973 cm^{-1} which indicates the C-H strain. Typical absorption on PVC material is found at the peak of the wave number of 1413 cm^{-1} and 675 cm^{-1} indicating the CH_2 and C-Cl functional groups. The C-O group in the wave number with a range of 1111-1043 cm^{-1} and the C-H functional group at the wave number 2937 cm^{-1} are derived from glycerol material. The functional group with a peak at the wave number of 3365 cm^{-1} indicates the -OH functional group. The amine functional group (NH_2) which is owned by the chitosan material is shown by the absorption wave number of 1647 cm^{-1}.[20]

The C–H trans functional group has a wave number of about 993 cm^{-1} and there is a C-Cl stretch with an absorption wave number of 675 cm^{-1} which comes from PVC material. In PVC-glycerol-chitosan biocomposite membranes, mixing between PVC material and chitosan material allows partial chain interactions or what is called dipole-dipole interaction between C-N bonds in chitosan and C-Cl bonds in PVC [20]. This kind of interaction may occur during the mixing process of the two solutions so that a mixture of PVC-chitosan has been obtained with several distributions between PVC and chitosan chains. The distribution of PVC and chitosan chains is influenced by the homogeneity of the solution, homogeneity or homogeneity is obtained in the process of mixing the two materials between the PVC solution and the chitosan solution that does not experience clumping and the mixing of the two solutions. The mixing of the two materials leads to the homogeneity of the solution.[20]

Scanning Electron Microscopy (SEM)

The observed concentration was 2.5% and 3% because the tensile test result met the standard. The sample of 2.5% PVC-Glycerol-Chitosan biocomposite membrane with a concentration of 2.5% shown in Figure 2 that have no pores but white spots or rough structure of the membrane caused by the bubbles in solution during printing process. The results of the Scanning Electron Microscopy (SEM) test of the PVC-Glycerol-Chitosan biocomposite membrane with a concentration of 3% shown in Figure 3 have a flat membrane structure and no pores indication. 3 % concentration PVC-Glycerol-Chitosan bio composite membrane has potential as a candidate for blood bag applications because it has no pore and has a smooth surface structure.

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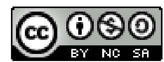

(A)

(B)

(C)

Figure 2. Result of Scanning Electron Microscopy (SEM) Test PVC-Glycerol-Chitosan biocomposite membrane with 2.5% concentration (A) 3,000X magnification, (B) 5,000X magnification, (C) 10,000X magnification.

(A)

(B)

(C)

Figure 3. Result of Scanning Electron Microscopy (SEM) Test PVC-Glycerol-Chitosan biocomposite membrane with 3% concentration (A) 3,000X magnification, (B) 5,000X magnification, (C) 10,000X magnification.

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In the Scanning Electron Microscopy (SEM) test results, the PVC-Glycerol-Chitosan biocomposite membrane with a concentration of 3% has a flat membrane structure and does not show any pores. The PVC-Glycerol-Chitosan biocomposite membrane sample with a concentration of 2.5% showed no pores but there were white spots or rough structure on the membrane caused by the bubbles in solution during the molding process.

The absence of pores or no pores on the PVC-Glycerol-Chitosan biocomposite membrane is due to the addition of plasticizer material to the PVC solution. According to Xu et al[21], the results of a membrane morphology test with a high plasticizer content equal to the amount of polymer solution or PVC can form pores because the plasticizer material prevents PVC polymer chains from forming a polymer matrix. One of the functions of the plasticizer is to act as a lubricant to allow the molecules in the plasticizer to be free from each other. Also to act as a partial solvent of polymer and can prevent pores in the membrane.[21] In accordance with this study, the ratio of the same or 1:1 between PVC and plasticizer resulted in the membrane becoming porous, so we used a ratio of 5:1 on the PVC and plasticizer solution, so as not to cause pores on the membrane. The increase in the ratio of 1:1 to 5:1 was due to the results in the ratio of 2:1, 3:1, and 4:1 in my research, which resulted in the membrane being porous and having a non-fine structure. Plasticizer in the application of blood bag membranes uses glycerol.

Hemolysis Test

The hemolysis test (shown in Figure 4) was carried out to determine the hemocompatibility of the PVC-chitosan-mangrove membrane so that it knew the cause of the red blood cell lysis. Increasing the concentration of chitosan can reduce the hemolysis properties of a material or membrane, and increase hemocompatibility in the membrane in accordance with the research conducted.[22] The hemolysis test results on PVC-glycerol-chitosan biocomposite membrane samples can interact with blood or not undergo hemolysis because the hemolysis percentage results are below 5%[23], so PVC-Glycerol biocomposite membranes with chitosan addition are hemocompatible and allow biocomposite membranes PVC-Glycerol-Chitosan to be applied as a blood bag.

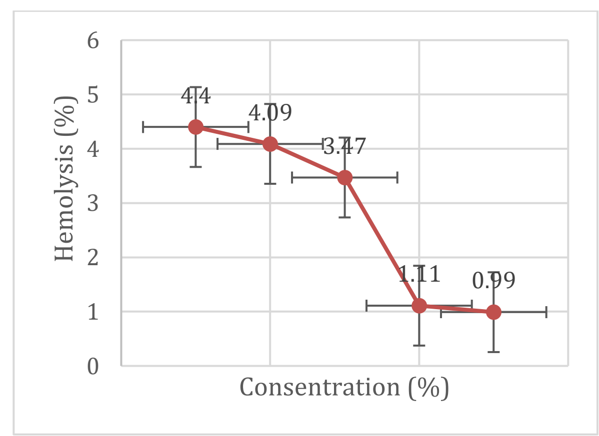

Figure 4. Results of Hemolysis Test

Antibacterial Tests

Antibacterial tests were conducted to determine the ability of PVC-Glycerol-Chitosan biocomposite membranes to inhibit bacterial growth. This test was carried out using two bacteria, namely *S. aureus* representing Gram-positive bacteria, and *E. coli* representing Gram-negative bacteria.

Analysis of the bacterial test was carried out by observing the inhibition zone. Measurement of the bacterial inhibition zone was carried out by measuring the diameter of the hole. The hollow formed is where bacterial growth is inhibited by the PVC-glycerol-chitosan biocomposite membrane, measuring the hollow using a caliper or ruler.

Antibacterial inhibitory power categorized as weak indicates a < 5mm inhibition zone, permitted to show a 5-10 mm inhibition zone, strongly supported showing a 10-20 mm inhibition zone, and proven to be

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very strong if using an inhibition zone of more than 20 mm. Antibacterial tests were carried out three times for each type of bacteria with variations in the concentration of chitosan 1.5%, 2%, 2.5%, and 3%, and this test used 0.5 MacFarland. The test results of PVC-glycerol-chitosan biocomposite membrane bacteria have been stated in Figure 5. and Table 1.

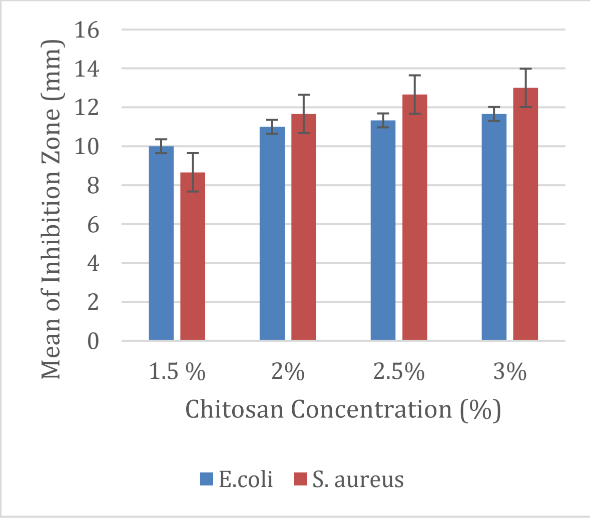


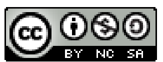
Figure 5. Results of Inhibition/Clear Zone (diameter) in Antibacterial Test

Table 1. Results of Inhibition/Clear Zone

Chitosan Concentration (%)	Mean of Inhibition Zone (mm)	
	E.coli	*S. aureus*
1.5%	10 ± 1,73	8.66 ± 0,55
2.0%	11 ± 1,73	11.66 ± 0,55
2.5%	11,33 ± 0,55	12.66 ± 0,55
3.0%	11.66 ± 0,55	13 ± 0,55

The bacterial test results of the PVC-glycerol-chitosan biocomposite membrane with a chitosan concentration of 1.5% had a bacterial inhibition zone diameter of 10 mm in *E.coli* bacteria and *S.aureus* bacteria had a diameter of 8.66 mm. Chitosan concentration of 2% showed an increase in the inhibition zone with a diameter of 11 mm in *E.coli* bacteria and an inhibition zone diameter of 11.66 mm in *S.aureus* bacteria. chitosan concentration of 2.5% had a diameter of 11.33 mm bacterial inhibition zone for *E.coli* bacteria and *S.aureus* bacteria had an inhibition zone diameter of 12.66 mm and at 3% concentration the diameter of inhibition zone for *E.coli* bacteria was 11.66 mm and *S.aureus* bacteria has an inhibition zone diameter of 12.66 mm, so the PVC-glycerol-chitosan biocomposite membrane bacterial test has strong antibacterial criteria but at a chitosan concentration of 1.5% in *S.aureus* bacteria it is categorized as weak because the diameter of the bacterial inhibition zone does not enter the strong category criteria.

The relationship of chitosan as an antibacterial depends on its affinity. The mechanism of very strong chitosan is with microbial DNA so that it can bind to DNA which then destroys mRNA and synthesis proteins. The antimicrobial affinity of chitosan in fighting bacteria or microorganisms depends on molecular weight and degree of deacetylation. Molecular weight and a greater degree of deacetylation show greater antimicrobial activity. Chitosan has a positively reactive, positively charged amine ($-NH_2$) functional group, so it can bind to negatively charged bacterial wall cells.[24] The potential of chitosan as an antibacterial agent is based on the initial interaction between chitosan and bacteria involving electrostatics. Chitosan has a positively reactive, positively charged amine ($-NH_2$) functional group, so it is able to bind to negatively charged bacterial wall cells. This bond occurs at the electronegative site on the surface of the bacterial cell wall. In addition, because $-NH_2$ also has a free electron pair, this group can attract Ca2+ minerals found in bacterial cell walls with covalent bonding. Changes in the cell surface and loss of protective function in bacterial cells leads to a reduction in the number of bacterial cells. Gram negative bacteria with lipopolysaccharide in their outer layer have a negative pole which is very sensitive to chitosan. However, the antibacterial activity of chitosan varies and is influenced by many factors such as molecular weight, pH value, and water solubility.[25]



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Tensile Strength Test

The Tensile Strength test was carried out at the ULP Faculty of Physics, Malang. The sample was prepared by a size of 6cm x 1cm in a rectangular sample using a paper holder and the IMADA tensile test instrument with a load cell of 50N. The results of tensile strength characterization obtained values of PVC-glycerol-chitosan biocomposite membrane (as shown in Table 2).

Table 2. Tensile Strength Test Result

Chitosan Concentration %	*Elongation Percentage* (%)	*Tensile Strength* (MPa)
0	7,976 ± 2,535	9,046 ± 1,796
1,5	6,580 ± 2,085	11,480 ± 0,797
2	5,154 ± 1,682	14,055 ± 2,725
2,5	4,854 ± 1,448	18,979 ± 2,451
3	3,510 ± 3,653	21,202 ± 2,849

A Tensile Strength test was conducted to determine the tensile strength of PVC-glycerol biocomposite membranes by the addition of chitosan and coating of mangroves *(Aegiceras corniculate)*. The results of the tensile strength characterization in tile strength can be seen in the greater concentration of chitosan, the greater the value of the tensile strength membrane. The increase in tensile strength is in line with the increase in the concentration of chitosan and this is related to the increase in hydrogen bonding formed in plastic films.[26] The PVC-Glycerol biocomposite membrane with the addition of chitosan, makes the formed bonds stronger and harder to break. The standard of tensile strength in a blood bag based on PVC material is 14-26 MPa[18], it is in accordance with the standard of blood bag tensile strength with the concentration of chitosan 2 wt/v%, 2.5 wt/v% and 3 wt/v%.

CONCLUSIONS

The addition of chitosan concentration can increase the physical and mechanical properties (tensile strength) of blood bags. The increase of chitosan concentration in the composite of PVC-Glycerol-Chitosan can meet the standards of tensile strength of the ideal blood bag membrane.

In the future the flexibility, and the condition under the thermogravimetric analysis to show weight loss in relation to alteration in the temperature of the membrane.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

Designed the study, synthesized, characterized, collected and analyzed the data : PW and AB. Manuscript preparation and writing : PW and TDPW. Manuscript correction : PW and S.

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Original Article

Measurements and Accuracy of IgM and IgG Anti Phenolic Glycolipid-1 Levels in Blood Serum for Early Detection *Mycobacterium leprae* by using Enzyme-Linked Immunosorbent Assay (ELISA): A Reality of a Laboratory

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ABSTRACT

Indonesia was the third most recent case of leprosy globally in 2020 with 11,173 people, after India and Brazil. Most of the leprosy manifestations are asymptomatic. This is possibly as subclinical leprosy which individuals without leprosy symptoms but have leprosy specific antibodies high levels, so it has the potential to become a transmission and disability. Therefore, an ELISA test need for early detection in preventing leprosy transmission. This study aims to measure IgM and IgG antibody levels in leprosy patients and assess the accuracy of the measurement results. This research is a cross-sectional study. Five patients' blood samples have analyzed for IgM and IgG anti-PGL-1 antibody levels by ELISA. Accuracy interpretation of this measurement based on the %CV. Antibody levels were classified based on the cut-off <605 u/ml as IgM seronegative or <630 u/ml as IgG seronegative, 605–1000 u/ml as low seropositive IgM or 630-1000 u/ml as low seropositive IgG, and >1000 u/ml as high seropositive IgM and IgG. Among five patients examined, 40% had high seropositive leprosy with anti-PGL-1 IgM and IgG antibody titers>1000 u/ml, and 60% of patients had seronegative leprosy. Accuracy in this ELISA test shows high accuracy with %CV <10% in the conversion of OD to antibody titer levels. IgM and IgG Anti PGL-1 antibody titers by ELISA as one of the parameters in identifying patients at higher risk of leprosy. A significant portion of patients with high seropositive leprosy with high accuracy.

Keywords: leprosy, early detection, ELISA, Anti-PGL-1, seropositive.

Highlights: The ELISA test can detect the presence of Anti Phenolic Glycolipid-1 (PGL-1), specific for *M. leprae.* Therefore, sensitive and specific early detection tool by ELISA anti-PGL-1 needs to be able to control the transmission of *M. leprae*

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INTRODUCTION

Leprosy or Morbus Hansen is one of the Neglected Tropical Diseases (NTD) with a chronic infection caused by *Mycobacterium leprae*. Leprosy has been found for thousands of years BC, it is still endemic in several countries, including Indonesia.[1] Based on data from the World Health Organization (WHO) for 2020, Indonesia ranks third with the most recent cases of leprosy globally with 11,173 people, after India and Brazil.[2] *M. leprae* first attacks the peripheral nerves where it can then attack the skin, upper respiratory tract mucosa, and tends to cause defects, especially in the organs of the hands and feet. Most of the manifestations of leprosy are asymptomatic, with clinical signs and symptoms in leprosy patients usually seen after five years incubation periods. Leprosy sufferers begin to experience white and red spots on the skin, a feeling of tingling, and organ dysfunction.[1]

Most of the leprosy control still focused on clinical leprosy following the recommendations given by WHO. Multi-Drug Therapy (MDT) in Indonesia has existed since 1980.[3,4] Healthy individuals have a natural immune system capable of fighting *M. leprae* infection, and only 15% of individuals with a weak immune system may become infected with *M. leprae*.[1] This is considered as subclinical leprosy in individuals who look healthy without any clinical signs and symptoms of leprosy but have high levels of specific antibodies against leprosy bacilli. Subclinical leprosy allows the development of clinical leprosy within 2-10 years later. Poor management of subclinical leprosy cases can potentially become a source of transmission and disability.[3–7] Therefore, sensitive and specific early detection tool needs to be able to control the transmission of *M. leprae*.

In addition to the presence of clinical symptoms, detections of antibody levels through the Enzyme-Linked Immuno-Sorbent Assay (ELISA) can show the activity and classification of the patient's current or previous infection. The ELISA test was introduced by Brett et al., in the 1980s.[8] ELISA is a well-based diagnostic test especially used in the study of immunology, that measures the concentration of antigens and antibodies in a sample through enzymes as markers. The ELISA test can detect the presence of Anti Phenolic Glycolipid-1 (PGL-1),specific for *M. leprae*.[4] Anti-PGL-1 antigen levels in blood serum can represent the level of antibody response given by the body.[4,6,7,9] Presence Anti PGL-1 is one of the biomarkers in identifying patients who are at higher risk of experiencing a reaction before the appearance of clinical manifestations of leprosy so that it can determine the best case management approach in preventing further disability and disease transmission as early as possible, especially in endemic areas.[6,9,10] Epidemiologically, 15% of leprosy cases in endemic areas are in the group experiencing a subclinical infection.[11] Research by Iswahyudi and Sujagat showed that 29.5% of subclinical leprosy cases in children were detected through ELISA antibody measurements.[12,13] Besides that, antibody measurements using for follow-up evaluation of treatment, which was accompanied by a decrease in circulating antibody titers.[14]

In general, the ELISA test consists of the stages of coating, blocking, carrying out several items of washing, incubation, carrying out several washing processes again, staining, stopping the staining process, and finally reading the optical density (OD) with a spectrophotometer. OD will be converted into antibody levels in units per milliliter by the Biolise software. Classifications of the antibody threshold value are determined based on the percentage of 80-90% of the lowest antibody level results.[14] The cut-off value of IgM Anti PGL-1 is 605 u/ml, and the cut-off value of IgG Anti PGL-1 is 615 u/ml what it has used as the classifications of a

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patient's results are included in leprosy seropositive or seronegative. The ELISA serological test can detect antigen and antibody concentrations at the level of 0.01 µg/ml with high specificity that can correctly identify people who do not have the disease at an ability level above 80%.[15]

MATERIAL AND METHODS

Materials

The tools used to research the detection of *M. leprae* by the ELISA test using blood serum samples include Immunowash (BIORAD model 1575), microplates, micropipettes, and tips with a size of 50 µl – 1000 µl, vortex, spindown, Biolise/X-read, Eppendorf tube of 1.5 µl, and incubation contacts. The materials used include 0.5 ml blood serum in a capillary tube, NT-P-BSA, Coating Buffer pH 9.6 ($NaHCO_3$, Na_2CO_3, NaN_3, Distilled Water), Phosphate Buffered Saline (Na_2HPO_4, KH_2PO_4, NaCl, KCl, Distilled Water), PBST (PBS, Tween20), Blocking Buffer (1% Skimmed Milk, NaN_3, PBS), Washing Buffer (PBST, Distilled Water), Stopping Solution (H_2SO_4 pk, Distilled Water), and Substrate Solution (Citrate-phosphate buffer, Ortho Phenylene Diamine, 30% H_2O_2). NT-P-BSA is a synthetic form of the anti-PGL-1 *M. leprae* whose manufacture and distribution is regulated by T. Fujiwara from Institute for Natural Science, Nara University.

Methods

Sample. This research is a descriptive study with a cross-sectional design. Five patient blood samples have been analyzed for IgM and IgG anti-PGL-1 levels by ELISA at the Leprosy Laboratory, Institute of Tropical Disease, Airlangga University, Surabaya for identifying patients with seropositive or seronegative status of leprosy. The five blood samples of the patients have been coded in the sample name IF, DT, SR, AS, and SO. Blood samples had centrifuged to separate serum to use in the ELISA test. The blood serum separation has transferred into a new 0.5 ml tube. Sampling was carried out using non-probability purposive sampling. The inclusion criteria in this study were a sample of suspected leprosy patients. Meanwhile, exclusion criteria included patients with other chronic infectious diseases, patients with other acute infections, and or patients with resistance to anti-leprosy drugs.

Antigens. Blood serum was analyzed using Indirect ELISA quantitatively using synthetic anti-PGL-1 (NT-P-BSA).

Anti-PGL-1 antibody assay. This study used the indirect ELISA method quantitatively. The ELISA method had described in the work instructions for serological examination of leprosy (ELISA Anti-PGL-1).[16] Each serum dilution was tested in duplicate on an Anti-PGL-1-coated microplate and a control microplate without antigen. The microplate was coated by 50 µl NT-P-BSA and 50 µl Coating Buffer pH 9.6 according to the specified scheme for overnight incubation at 4^0C. The microplate has washed with PBST solution, then coated again with 200 µl of Blocking Buffer, and then incubated at 37°C for one hour. The blocking buffer was discarded, and 50 µl of serum was added to the microplate. Fifty µl of serum volume was diluted 1:300 in Dilution Buffer. Each standard and blank well was diluted in five different concentration ratios (0, 5, 10, 15, 20). Samples were tested in duplicate and incubated for one hour at 37°C. The microplate was washed, and 50 µl of secondary antibody IgM and IgG conjugated with the enzyme was added, which was diluted 1:2000 in Dilution Buffer. Microplate has incubated at 37°C for one hour. The microplates were washed and then stained with 100 µl of the substrate containing Ortho Phenylene Diamine (OPD) and 30% Hydrogen Peroxidase in Citrate-Phosphate Buffer. The microplate was incubated until a yellow or orange color developed. The staining reaction stopped after 10-30 minutes by adding 100 µl of Stop Solution containing

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sulfuric acid. The result of the ELISA test has read by using ELISA Reader at the wavelength of 492 nm/620 nm.

Statistics. ELISA results in Optical Density (OD) are presented in a standard curve to determine the relationship between concentration and absorbance. The regression line and the correlation coefficient in a standard curve had depicted in a 4-parameter regression. OD results can be converted to units/ml using the Biolise software to determine the levels of detected antibodies. The results had expressed as the average value of the antibody titer for each sample obtained based on the concentration and dilution factor in each sample well. Antibody levels in u/ml were classified using an IgM cut-off value of <605 u/ml as seronegative, 605–1000 u/ml as low seropositive, or >1000 u/ml as high seropositive, and an IgG cut-off value of <630 u/ml as seronegative, 630-1000 u/ml as low seropositive, or >1000 u/ml as high seropositive leprosy. The accuracy of the ELISA test in converting the OD results into concentrations levels (units/ml) is shown in the results of the percentage coefficient of variation (%CV) by Biolise software.

RESULTS AND DISCUSSION

Results Optical Density

The microplate schematic consisting of IgM and IgG Anti-PGL-1 schemes are analyzed simultaneously.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.066	0.009	0.009	1.299	0.149	0.099	0.039	0.029	0.034	0.418	0.168	0.488
B	0.330	0.328	0.318	1.231	0.133	0.097	0.330	0.345	0.346	0.454	0.170	0.477
C	0.771	0.770	0.742	0.100	0.100	0.088	0.502	0.512	0.463	0.197	0.174	0.489
D	0.870	0.868	0.811	0.096	0.101	0.098	0.767	0.779	0.739	0.195	0.157	0.529
E	1.113	1.085	0.981	0.442	0.069		0.923	0.940	0.883	0.203	0.381	
F	0.064	0.134	0.145	0.455	0.067		0.171	0.204	0.231	0.206	0.370	
G	0.075	0.128	0.149	0.138	0.057		0.150	0.204	0.236	0.217	0.412	
H	0.043	0.204	0.205	0.135	0.067		0.037	0.305	0.305	0.217	0.415	

Figure 1. Optical Density Result

All rows in the first to sixth columns are IgM schemes, while the seventh to twelfth columns are IgG schemes. The scheme consists of the standard in an orange well, the blank in a blue well, and the sample in a white well. Each sample has been analyzed in duplicate on both antigen and antibody coatings. Each standard and blank well has five different concentration ratios (0, 5, 10, 15, 20). The OD results are different for each sample well, standard, and blank.

The OD results on standard wells and well blanks were lower than the OD results on sample wells. The lowest OD results for standards and blanks were the standard and the blanks wells with a concentration of zero, namely 0.009/0.029 in standard wells and 0.043/0.037 in well blanks. The lowest OD result in the SO sample well was 0.057, and the highest in the IF sample well was 1.299.

Standard Curve

The standard curve consists of the X-axis showing the concentration level and the Y-axis showing the absorbance value. The X-axis stretches horizontally, and the Y-axis stretches vertically on the standard curve. The regression equation on the standard curve is $y = (a-d) / (1+(x/c)^b) + b$.

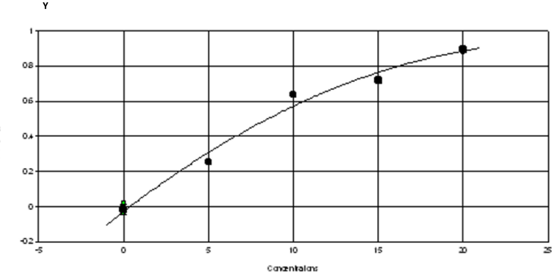

Figure 2. IgM PGL-1 Curve Standard

On the IgM standard curve with the equation $y = 0.001422x2+0.0745x–0.02879$ with an R^2 of 0.9830.

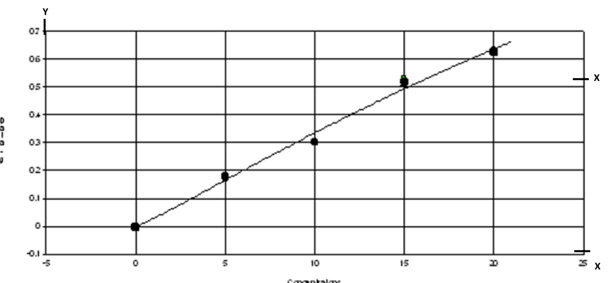

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Figure 3. IgG PGL-1 Curve Standard
On the IgG standard curve with the equation y = -2.6753693 / (3.675+(x/56.44)$^{1.123}$) with an R^2 of 0.9920.

IgM PGL-1 and IgG PGL-1 Antibody Titer Levels

IgM and IgG anti-PGL-1 in ELISA results are displayed in the form of a distribution which is classified based on level seropositive or seronegative.

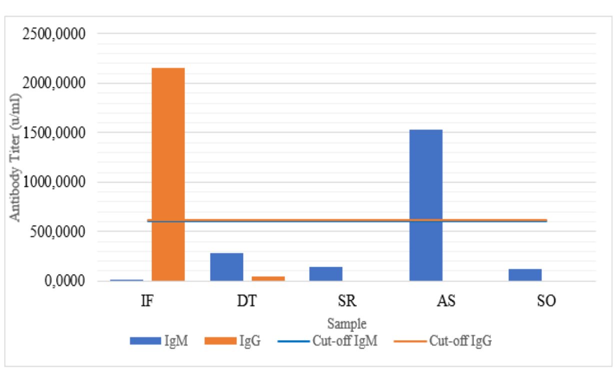


Figure 4. Distributions of Antibody Titer Levels

The results of the ELISA serology showed that out of all five samples tested, three blood samples showed seronegative leprosy, while the other two showed high seropositive leprosy in IgM anti-PGL-1 and IgG anti-PGL-1 antibody titers.

ELISA Test Method Accuracy

The accuracy of the ELISA test in converting the OD results into concentrations levels (units/ml) is shown in the results of the percentage coefficient of variation (%CV).

Table 1. Percentage CV of ELISA Test

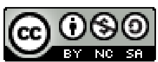
Stand.	Conc.	%CV	
		IgM	IgG
1	0	0	0
2	5	2.0176	3.8769
3	10	0.1045	1.6557
4	15	0.2077	2.9767
5	20	0	1.5772

Based on the percentage coefficient of variation of IgM and IgG standard solutions (<10%) shows that the ELISA test method used has high accuracy.[16]

Discussion

Early detection of *M. leprae* is important in preventing further disability and breaking the transmission chain, especially in cases of subclinical leprosy which often go undetected.[3,5–7] Phenolic Glycolipid (PGL-1) is the dominant component of carbohydrates and lipids in *M. leprae*. This antigen is found in all tissues infected with *M. leprae* and can survive until the tissue dies.[17] Although it can stimulate the body's immunity, PGL-1 is not enough against *M. leprae*. Therefore, the ELISA test had carried out by measuring antibody titers in blood serum against anti-PGL-1 as a specific antigen for *M. leprae* and was used to detect leprosy early.[18]

The reading of the ELISA test results is in the form of Optical Density in Figure 1. Each well has an optical density (OD) with different values and concentrations. These differences have been influenced by the number of antigen and antibody bonds formed using enzyme-labeled secondary antibodies as markers. Antigens with non-specific antibodies or vice versa will not form specific bonds. In the microplate schematic, the positions of rows A and the first until third column or the seventh until ninth column do not show any antigen and antibody reactions where the wells only contain antigens without antibody titers. The position of rows F, G, and H, and the first until third column or the seventh until ninth column only contains antibodies without antigens so that reactions with specific antigen-antibody bonds do not occur. In line with the results, the lowest OD results are well standard and well blank. The position of rows B, C, D, and E, and the first until third column or the seventh until ninth column show a reaction in which antigen, antibody, and enzyme-labeled secondary antibodies have formed in the wells as markers. In line with the results of Optical Density in Figure 1., the well samples had



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higher OD results than well standard wells and well blanks without a specific binding reaction between antigen and antibody.

Quantitative analysis of ELISA test results also compares the concentrations of antigens or antibodies in the sample by using the standard curve. OD results have been converted into a standard curve to determine whether there was an effect between the OD or absorbance value and the concentration. The concentration level has been obtained by the dilution factor of the standard solution, and the absorbance value has been obtained by the standard solution concentration. Regression standard curves for the corresponding absorbance values and concentrations of reference standard concentrations have shown at the R^2.[19] An R^2 curve has considered having the goodness of fit when the R^2 value is over 0.99.[20,21] R^2 value in the Figure 2. IgM Anti-PGL-1 standard is 0.9830, meaning the concentration value of the solution determined had influenced by the absorbance value of 98%. This value is considered insignificant to the relationship between the absorbance value and the concentration of the reference standard determined. R^2 value in the Figure 3. IgG Anti-PGL-1 standard is 0.9920, meaning the concentration value of the solution determined had influenced by the absorbance value of 99%. This value is considered significant to the relationship between the absorbance value and the concentration of the reference standard determined. The value of R^2 >0.99 shows a significant relationship between the absorbance value and concentration.[20,21] Thus, the interpretation of optical density results can be predicted accurately.

Each sample was analyzed in duplicate so that the sample antibody titer value was the average result of the sum of each multiplication between the concentration and the diluent factor in each sample well. Antibody titer value (unit/ml) is the result of conversion from OD using Biolise software to detect antibody titer values related to the presence or absence of *M. leprae* in the human body. When *M. leprae* manages to enter the body, Anti-PGL-1 will stimulate the immune system to produce antibodies against *M. leprae* has been recognized as foreign cells.[6] The IgM and IgG antibodies have produced by T lymphocyte cells that stimulate interferon and interleukin in activating the cellular immune response. T cells control the proliferation of B cells to produce IgM and IgG antibodies.[22]

When first infected, Anti-PGL-1 can stimulate the body's immune system to produce IgM antibodies. The IgM antibodies can appear after 1-2 weeks to 2-3 months after exposure to *M. leprae*.[23] The IgM antibodies to anti-PGL-1 indicate the patient has an acute immune response or is suffering from leprosy.[22,23] IgM Anti-PGL-1 antibody titer level can represent the bacterial load of *M. leprae* in the human body.[24,25] Patients whose seropositive IgM anti-PGL-1 titers do not correlate with clinical symptoms of leprosy indicate the possibility of subclinical infection.[24,25] This is due to the absence of likely cross-reactions between Anti-PGL-1 and other mycobacteria. In Figure 4. IgM and IgG PGL-1 Antibody Titer Levels, AS patients showed high seropositive results of IgM anti-PGL-1 titers above 1000 units/ml. The results of the ELISA serological examination in Figure 4. showed that the IgM anti-PGL-1 antibody titer in AS patients were 1528.1 units/ml, and the IgG anti-PGL-1 was 0 units/ml. The high titers of IgM antibody to anti-PGL-1 correlate with ongoing or recent infection in AS individuals. The IgM anti-PGL-1 antibody titers that exceed the threshold may indicate that leprosy infection in acute AS patients has a higher risk of developing leprosy manifestations in the next few years.[6,7,26] The association between anti-PGL-1 IgM positivity and the development of leprosy cannot imply that the anti-PGL-1

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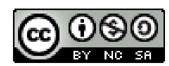

results reflect a recent infection with *M. leprae*. Laboratory tests on experimental animals showed that the response of anti-PGL-1 antibodies in leprosy patients has positively correlated with the bacillary burden of *M. leprae* in the body.[27] This correlation may indicate that anti-PGL-1 positive, healthy contacts have been exposed to *M. leprae* and have a high bacillary burden. This hypothesis is consistent with the fact that IgM antibodies exhibit an early response to infection. Nevertheless, animal models have shown that IgM antibodies can persist and participate in long-term protection against obligate intracellular bacteria.[28] In the research with animal models, IgM anti-PGL-I is still present at higher levels many years after infection.[29] These data corroborate the finding that IgM antibodies in human leprosy not only indicate recent infections. Interpretation of anti-PGL-1 IgM titer levels also requires the results of an anti-PGL-1 IgG examination. The tendency for low IgM anti-PGL-1 antibody levels to remain positive may be related to bacillary persistence. Persistence is possible due to the PGL-1 antigen can still stimulate the low antibody response in the absence of living *M. leprae* bacilli.[30,31]

Anti-PGL-1 also stimulates IgG antibodies which indicate an immune response against chronic disease. The IgG anti-PGL-1 antibody titer using as a biomarker that can detect and predict *M. leprae* infection retrospectively.[32,33] Exposure history of patients who don't suffer from leprosy, but whose been exposed to *M. leprae* before is indicated by the level of IgG antibody titer.[22] In Figure 4. IgM and IgG PGL-1 Antibody Titer Levels, IF patients showed high seropositive results of IgG anti-PGL-1 titers above 1000 units/ml. The results of the ELISA serological examination in Figure 4. showed that the titer of IgM anti-PGL-1 antibodies in IF patients were 0.7081 units/ml and IgG anti-PGL-1 was 2153.3 units/ml. The high IgG titer indicates that the IF patients had been exposed to *M. leprae* or have been in contact with leprosy patients for months or years. The bacteria load of *M. leprae* in the body can slowly decrease with efficacy patient treatment.[27] Although IgG antibody titer levels are still high, IgM anti-PGL-1 antibody titers that have been low during the first year of treatment can be used for treatment efficacy evaluation. Research by Touw, Bach, and Khadge showed a significant decrease in IgM anti-PGL-1 antibody titers compared to IgG anti-PGL-1 was observed within one year after treatment.[34]

The study by Douglas et al., (2004) showed that individuals who were anti-PGL-1 seronegative had 75 times smaller risk than seropositive contacts to infected leprosy.[35] In Figure 4. IgM and IgG PGL-1 Antibody Titer Levels, the results of anti-PGL-1 seronegative antibodies have shown in DT, SR, and SO patients. The result of seronegative leprosy had shown by the cut-off IgM anti-PGL-1 titer below 605 units/ml and the IgG anti-PGL-1 titer below 630 units/ml. The results of the ELISA serological examination in Figure 4. showed that the titer of IgM anti-PGL-1 antibodies in DT patients was 285.62 units/ml, and IgG anti-PGL-1 was 49,955 units/ml. The results of the ELISA serological examination in Figure 4. showed that the titer of IgM anti-PGL-1 antibodies in SR patients was 137.92 units/ml, and IgG anti-PGL-1 was 0 units/ml. The results of the ELISA serological examination in Figure 4. showed that the titer of IgM anti-PGL-1 antibodies in SO patients was 121.46 units/ml, and IgG anti-PGL-1 was 0 units/ml.

Quantitative results of the ELISA test can assess seroprevalence and monitor changes in transmission time and place of transmission of *M. leprae*, especially in endemic areas at higher accuracy levels.[36] The accuracy of this ELISA test method has been assessed by the percentage coefficient of variation (%CV) of the solution standard with a known concentration (5 different concentrations – 0, 5, 10, 15, 20).[16] The conversion of Optical Density (OD) by Biolise software into a standard solution whose concentration is known can show the percentage coefficient of variation.[37] In Table 1. showed that the first

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standard solution has a %CV IgM Anti-PGL-1 titer and IgG Anti-PGL-1 titer of 0%. In Table 1. showed that the second standard solution has a %CV Anti-PGL-1 titer of 2.0176% and an Anti-PGL-1 IgG titer of 3.8769%. In Table 1. showed that the third standard solution has a %CV Anti-PGL-1 titer of 0.1045% and an Anti-PGL-1 IgG titer of 1.6557%. In Table 1. showed that the fourth standard solution has a %CV Anti-PGL-1 titer of 0.2077% and an Anti-PGL-1 IgG titer of 2.9767%. In Table 1. showed that the fifth standard solution has a % CV IgM Anti-PGL-1 titer of 0% and an Anti-PGL-1 IgG titer of 1.5772%. There is no consensus for this accuracy determination. Most of the studies conducted use conventional ELISAs that are self-made, and due to other factors such as sampling, sample delivery, or sample preservation can cause variations in the accuracy of the assay.[38] Based on the percentage coefficient of variation, it shows that the conversion of absorption rates into units/ml has high accuracy at the %CV at the five concentration values being very small (below 10%). Research by Faizo et al. shows that the ELISA antibody detection protocol for SARS-CoV-2 uses the same consensus where %CV <10% indicates a high level of test accuracy.[39]

CONCLUSIONS

IgM and IgG Anti PGL-1 antibody titers by ELISA as one of the parameters can identify patients at higher risk of leprosy. The study results showed that a significant portion of patients has high seropositive leprosy with high accuracy. In the future, this test has been expected for early diagnosis of leprosy, especially in endemic areas so that leprosy does not develop into a transmission and further disability.

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CONFLICT OF INTEREST

All authors have no conflict of interest.

AUTHOR CONTRIBUTION

Designed the study, collected and analyzed the data, and also prepared the manuscript: SPKA and LNY. A scientific adviser in the field of leprosy and laboratory: DA, II, PAW, MD, RW, and CRSP. All authors read and approved the final manuscript.

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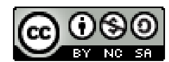

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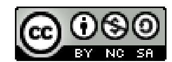

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Review Article

Different COVID-19 mRNA-based Vaccine Platforms as The Booster Dose and Their Impact on Omicron: A Literature-Based Overview

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ABSTRACT

Globally, the vaccine has been determined as one of the principal policies to tackle the COVID-19 pandemic. However, some vaccinated individuals with two complete doses of inactivated experienced SARS-CoV2 infection, including the healthcare workers (HCWs). This threat led to the emergent need for a vaccine booster with different types of platforms aiming to enhance immunity from the Omicron variant. We conducted a literature study on the concept of heterologous compared to homologous vaccines in COVID-19 vaccination. We obtained 22 studies about COVID-19 booster vaccines. Referring to seven of them, we compared and distinguished between heterologous and homologous vaccines. We then reported the literature review according to PRISMA guideline. The study demonstrated qualitatively that heterologous vaccinations boosted antibody receptor binding domain, neutralizing antibody, and spike-specific Th1 type T cell responses and had an impact on omicron infection when compared to homologous vaccines. In conclusion, heterologous, mRNA based vaccine, predominantly induces cellular and humoral responses better than the homologous vaccine. This increased immune response is expected to provide profound immunity against the Omicron.

Keywords: vaccine, COVID-19, infectious disease, heterologous, booster *vaccine, COVID-19, infectious disease, heterologous, booster*

Highlights: The combination of two different COVID-19 vaccine platforms with mRNA based vaccine platforms strengthens the immune response and is expected to be able to counteract the Omicron variant

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INTRODUCTION

In May-July 2021, various variants of SARS-COV-2 appeared, followed by its rocketing transmission in Indonesia. The Delta variant dominantly emerged.[1] Many patients were infected by this variant, including HCWs who previously received two complete doses of vaccination. Due to the surge of breakthrough infection even after completing two doses of vaccine, as recommended by the National Immunization Expert Advisory Committee or ITAGI, giving the third dose of vaccination was considered necessary.[2,3]

Our prior study confirmed that health care providers (HCPs) were susceptible to breakthrough infection, specifically them with hypertension. The most effective vaccines, by far, are known to increase the production of neutralizing antibodies which will later prevent infection. One of the strategies implemented is heterologous prime-boost vaccination.

Several previous studies have proven this method is more effective in enhancing vaccine action in preclinical studies. However, research on humans' immune responses using this method is still being carried out.[3] Heterologous prime-boost vaccination is a vaccine method by inserting the same nucleotide or antigen expressed by different vectors for primary or booster/repeat vaccination. According to WHO (2021), other reasons for using heterologous vaccines include reducing vaccine adverse reactions, increasing immunity to the SARS-CoV-2 virus, and strengthening vaccine effectiveness. Prior research on the heterologous prime-boost vaccine, in particular the combination of exogenous (inactivated vaccines) and endogenous (mRNA vaccines), had demonstrated considerable improvements in the immunogenicity of the HIV-1, influenza, and particularly the SARS-CoV-2 vaccines.[4,5]

As above mentioned, the use of a heterologous vaccine for booster dose is to anticipate the emergence of SARS-CoV-2 infection from various variants, especially Omicron, that is so contagious.[6-13] By far, several studies on the effect of booster vaccine in preventing Omicron infection have shown varying results. There are no clear studies stating whether to use homologous or heterologous vaccines for omicron variations.[14-16] In Indonesia, this condition is a dilemma because vaccine availability is also limited there are no clear references that compare the two types of vaccines. In this study, we reviewed preceding literature about the administration of booster vaccine with two different platforms and how it prevents Omicron infection so that we are right in giving vaccine boosters.

MATERIALS AND METHODS

Materials

We performed an electronic literature search from PubMed, Springer, and the Cochrane Library to identify studies exploring the use of heterologous COVID-19 vaccine regimen. The keywords used were (heterologous) AND (prime-boost) AND (inactivated) AND (SARS-CoV-2) AND (Omicron) AND (vaccine) AND (neutralizing antibody) AND (T cell response) AND (IgG subtypes). The last search was conducted from November 21st 2021 until June 30th 2022.

Methods

Protocol trial, review, comparative study, experimental study, case report, pre proof, and systematic review were eliminated. The relevant studies were collected and screened as shown in Figure 1 and Figure 2.

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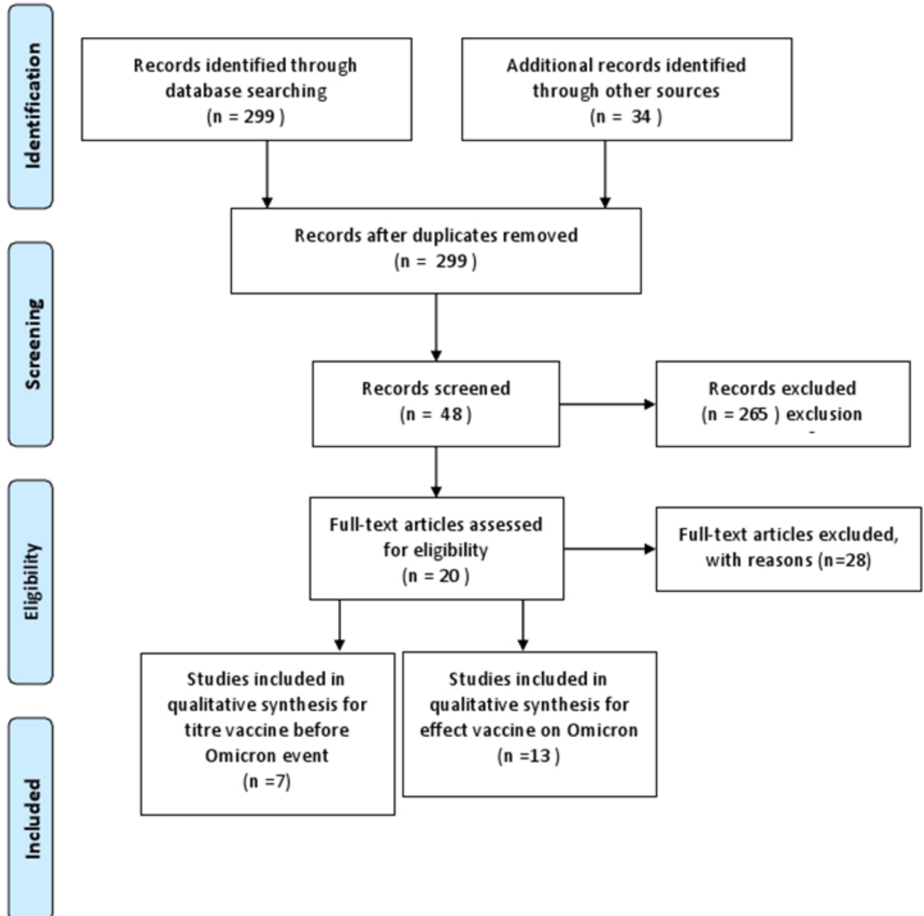

Figure 1. Literature Selection

We made a descriptive comparison between immunological parameters after heterologous and the homologous vaccine. We included the author's name, the year of study, the country of research and publication, the types of vaccine, and the final result. We give (+++) for an increase in immunological parameters more than >100x; (++) if 50-100x; and (+) when <50x.

RESULTS AND DISCUSSION

We derived 20 studies from the literature search and summarized them in Table 1 and 2. Of these subsequent studies, the mRNA vaccine was mostly used as the heterologous booster vaccine. Table 1 compares the immune response after heterologous vaccine with the homologous vaccine. Seven studies showed that heterologous vaccines provided an enhanced receptor-binding domain (RBD) antibody, neutralizing antibody, and spike-specific Th1 type T cell responses better than homologous vaccines.

Thirteen studies above stated that booster vaccines, both heterologous and homologous, boosted protection against Omicron variants, despite decreasing neutralizing antibody titers. Heterologous booster provides superior protection compared to homologous ones in preventing Omicron infection. Cheng study in 2022 stated that the majority of people receiving three CoronaVac treatments by failed to produce Omicron-neutralizing antibodies.

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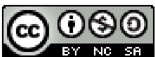
Table 1. The comparison of immune response between heterologous vaccine and homologous vaccine before Omicron.

Study	Platform vaccine	Heterologous			Homologous		
		Antibody RBD	Antibody Neutralizing	Spike T cells	Antibody RBD	Antibody Neutralizing	Spike T cells
Atmar et al. 2021	mRNA/mRNA vs mRNA/viral Vector	+++	+++	Not exam	++	++	Not exam
Xinxue Liu et al. 2021	Chad/ChAd or BNT/BNT vs Chad/BNT or BNT/Chad	+++	+++	+++	++	++	++
Joana Barros-Martins et al. 2021	ChAd/BNT vs ChAd/ChAd	+++	+++	+++	++	++	++
Tensbuch et al. 2021	ChAdOx1 nCoV-19 / BNT162b2	Not exam	+++	Not exam	Not exam	++	Not exam
Kant et al. 2021	ChAdOx1/ChAdOx1 vs ChadOx1/ BBV152 ChadOx1	+++	+++	Not exam	++	++	Not exam
Benning et al. 2021	ChAdOx1 nCoV-19 / BNT162b2	++	++	Not exam	++	++	Not exam
Hilus et al. 2021	BNT/BNT or ChAdOx/ChadOx vs ChAdOx/BNT	++	++	+++	++	++	++

Table 2. Effect vaccine booster for omicron event.

Author	Vaccine booster	Effect to Omicron
Ai et al., (2022)	BBIBP-CorV vs ZF2001	Reduced potency of geometric mean neutralizing titers (GMTs), higher GMTs in heterologous booster group.
Wang et al, (2022)	BBIBP-CorV vs ZF2001	Homologous or heterologous vaccine reduces the omicron escape from neutralization even though the levels are decreased.
Poh et al., (2022)	mRNA-1273 vs BNT123b2	A stronger neutralizing response to the Omicron variant was induced by the heterologous mRNA-1273 booster vaccine in older people than by the homologous BNT123b2 vaccine.
Zuo et al., (2022)	inactivated vaccine (CoronoVaccine, BBIBP-CorV) vs mRNA (BNT162b2, mRNA1273)	In people who have received two doses of an inactivated vaccine and a booster dose of an mRNA vaccine, the levels of specific antibodies, responses from memory B and T cells, and neutralization activities against the SARS-CoV-2 virus and VOC, including the novel Omicron form, have significantly increased.
Wang et al., (2022)	Inactivated vaccine against RBD recombinant subunit vaccine (Zifivax) (I-I-S) (CoronaVac or BBIBP-CorV)	In comparison to homologous booster(I-I-I), heterologous booster (I-I-S) has a greater ability to neutralize various VOCs, including omicron.



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Author	Vaccine booster	Effect to Omicron
Du et al., (2022)	Recombinant protein subunit vaccines, inactivated vaccines, vector vaccines, and mRNA vaccines (BNT162b2 and mRNA-1273), as well as inactivated vaccines (BBIBP-CorV and CoronaVac) (ZF2001 vaccine)	The mRNA vaccinations were generally very effective against the Omicron variety, especially the mRNA-1273 vaccine. Furthermore, it didn't seem like heterologous booster immunization regimens were worse than homologous booster vaccination regimens.
Au and Cheung (2022)	mRNA vaccine (BNT162b2 vaccine and mRNA-1273 vaccine), inactivated vaccine (BBIBP-CorV and CoronaVac), vector vaccine (ADZ1222 vaccine and Ad26.COV2.S vaccine)	Three dosing regimens of homologous and heterologous drugs effectively reduce omicron infection. Any original vaccine that includes an mRNA booster provides high levels of protection comparable to a three doses mRNA regimen.
Suah et al., (2022)	BNT162b2, CoronaVac, and AZD1222	Homologous BNT162b2 boosting was less successful than heterologous boosting for CoronaVac and AZD1222 primary immunization patients.
Cheng et al., (2022)	CoronaVac or BNT162b2	Homologous or heterologous booster doses of BNT162b2 improve neutralizing antibody levels against the Omicron variety after two doses of either CoronaVac or BNT162b2. Most participants took three doses of CoronaVac without producing any Omicron-neutralizing antibodies.
Fang et al. (2022)	mRNA vaccine	After a single dose in animal models, the heterologous Omicron LNP-mRNA booster induced a more potent anti-Omicron antibody response than the WT booster.
Ai et al., (2022)	homologous booster group for BBIBP-CorV and a heterologous booster group for BBIBP-CorV/ZF2001	A marked decline in pVNT titre against Omicron after 14 days following booster doses of homologous or heterologous vaccine when compared to the prototype. When compared to the BBIBP-CorV homologous group, the GMT of the BBIBP-CorV/ZF2001 heterologous group was significantly higher.
Perez-Then., et al (2022)	CoronaVac plus BNT162b2	In comparison to the original strain and the Delta variation, neutralizing antibody titers for Omicron were decreased by 7.1-fold and 3.6-fold, respectively.
Costa Clemens et al., (2022)	A third homologous dose of CoronaVac vs a recombinant adenoviral-vectored ChAdOx1 nCoV-19 vaccine (AZD1222, AstraZeneca), an mRNA vaccination (BNT162b2, Pfizer-BioNTech), or an mRNA vaccine (Ad26.COV2-S, Janssen).	The live virus neutralization titres against both the delta and omicron versions are increased by heterologous boosting. After an mRNA spike, the highest antibody concentrations are seen.

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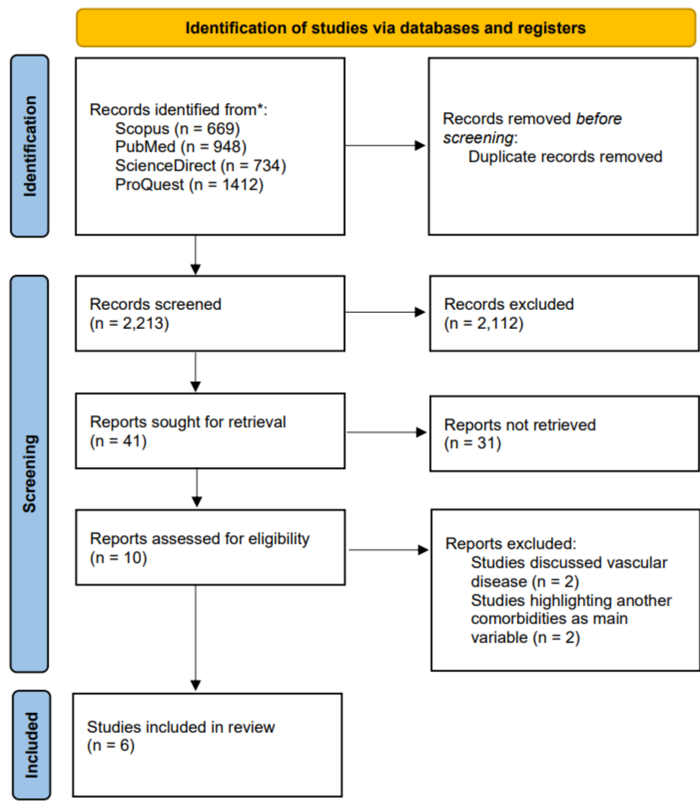

Figure 2. Schematic Workflow of Studies' Finding.[11]

Table 3. Characteristics of Selected Studies

Author	Year	Location	Sample Size	Ab	Vaccine	Dose	Measurement (Weeks after Dose 2)	Age (Years)	Male (%)	BMI (kg/m^2)	Hyper-tension (%)	Dia-betes (%)	Smokers (%)
Watanabe et al[13]	2021	Japan	68	IgS	BNT162b2	2	1-4	29.0 (17.0)	39.5	22.4 (5.5)	15.3	2.4	31.7
Ebinger et al[14]	2022	USA	843	IgS	BNT162b2	2	1, 2, 8, 16, 24, 32, 40	45.0 (13.0)	30.0	-	15.2	-	-
Delgado et al[15]	2022	Spain	2174	IgS	BNT162b2	2	12	45.9	19.9	24.1	8.1	-	22.2
Soegiarto et al[16]	2022	Indonesia	101	IgG	CoronaVac	2	4, 12, 20	47.7 (18.9)	59.5	-	23.7	17.8	10.9
Parthymou et al[17]	2022	Greece	712	IgS	BNT162b2	2	3, 12	50.8 (11.4)	37.6	26.7 (4.9)	16.2	7.0	34.4
Rifai et al[18]	2022	Indonesia	155	IgG	CoronaVac	2	8, 24	39.0 (9.2)	48.3	27.9 (7.3)	18.7	-	-

Table 4. Results of Selected Studies

Author	Vaccine	Results
Watanabe et al[13]	mRNA	Hypertensive patients presented lower antibody response compared to normotensive (650 ± 1192 vs 1911 ± 1364, p = 0.001). Hypertensive patiens shown significant beta coefficient on univariate and multivariate analysis with -1033.16 (p = 0.005) and -973.27 (p = 0.036) respectively.
Ebinger et al[14]	mRNA	Hypertensive patients shown significant beta coefficient on multivariate analysis with -0.17 and SE of 0.08 (p = 0.041).
Delgado et al[15]	mRNA	Hypertensive patients shown insignificant fold changes with -1.02 (p = 0.8584).
Soegiarto et al[16]	Inactivated	Hypertensive patients shown significant beta coefficient on multivariate analysis with -11.208 (p = 0.038). Patients with history of cardiovascular diseases shown non-significant beta coefficient on multivariate analysis with -10.040 (p = 0.969)

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Parthymou et al[17]	mRNA	Hypertensive patients shown insignificant beta coefficient on multivariate analysis with -0.0454 (p = 0.3276).
Rifai et al[18]	Inactivated	Patients with high systolic blood pressure and high diastolic blood pressure shown significant correlation with lower antibody response with R coefficient of -0.172 (p = 0.016) and -0.139 (p = 0.043) respectively second months after vaccination, and R coefficient of -0.284 (p = 0.046) and -0.475 (p = 0.006) respectively six months after vaccination.

The Dynamics of Antibody Level Following Homologous vs Heterologous Vaccine

The development of vaccines currently focuses on maximizing the immune response targeting RBD. It is assumed that antibodies bound to this domain can prevent the virus from entering the host cell. Other epitopes of protein S can also be targets of vaccines that can produce significant effects. Polyclonal antibodies against protein S epitopes besides RBD may also inhibit viral binding.[17]

According to a prior study, 88-97% of participants who got the second dosage of CoronaVac at 14-day intervals had antibodies that selectively bind to RBD on day 28 after treatment. Meanwhile, in the 28-days interval group, 92-100% of participants had an increase in RBD-specific binding antibodies. Furthermore, neutralizing antibodies were detected in all participants 21 days following the second dose of CoronaVac.[18,19]

Selecting a booster vaccine with variable work mechanisms (heterologous) is expected to increase the immunity against SARS-CoV-2 virus infection. Research on the administration of the third dose of Moderna has also begun to determine its effectiveness by measuring antibody titers. An observational study was conducted on a group of healthy adults in Germany who used a combination of the ChAdOx1 nCoV-19 vaccine (AstraZeneca), an mRNA booster vaccine, and BNT162b2 (Pfizer) or mRNA-1273 (Moderna). Of the 216 subjects, the participants were divided into 3 groups; 97 subjects in the heterologous group (AstraZeneca - Pfizer/Moderna), 55 subjects in the homologous AstraZeneca group, and the mRNA homolog group with 62 subjects involved.[20]

The results of the heterologous vaccine group, in which mRNA was used as the third dose, showed that the concentrations of spike-specific IgG protein, neutralizing antibody, and spike-specific CD4 T cells were significantly higher than the AstraZeneca homolog group or mRNA. CD8 T cell levels were also significantly higher in the heterologous vaccine group.[20] Researchers performing a similar experimental study concluded that the heterologous vaccines can generate stronger humoral and cellular immune responses against SARS-Cov-2 infection with the sufficient reactogenicity profile.[20-23]

Zhang's study in 2021 was conducted on a group of mice with immune characteristics after the third booster with various types of vaccines. Previously, the group of rats had been given two inactivated virus (INA) vaccines. Humoral and cellular immune responses (T cells) were observed after administration of recombinant RBD vaccine (rRBD), Ad5-vectored adenovirus (rAd), mRNA vaccine and INA vaccine. Neutralizing antibody (NAb), which targets the spike protein, was also observed in the mice group. This study concluded that the heterologous vaccine, a combination of INA with booster rRBD, rAd, and mRNA, increased NAb antibody titres and Th-1 type T cell response. The mRNA and rAd vaccines showed the highest NAb titers and T cell responses. The increased response of Th-1 cells can be seen from the high levels of IFN-γ and IL-2.[21]

Other studies showed that increase in RBD, Nab, and spike T-cell responses was observed after mRNA vaccine as the booster for adenovirus vaccine in the majority of the adult population, especially in healthcare

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workers.[20,24-31] Kant *et al.* (2021) revealed that administering inactivated virus and viral vector vaccine induced high neutralizing immune response against alpha, beta, and delta variant of SARS-COV-2.[32]

Good Responders vs Non/Less Responders

According to numerous research, those over 60 are more likely to have COVID-19 infection and experience worse outcomes, especially those who already have coexisting illnesses. Typically, this risk increases with age.[33-35] Older individuals are less responsive to the vaccine due to the aging of immune cells. The innate and adaptive immune systems' cellular and molecular components can be affected by modifications associated with aging in general.[35,36] Various types of vaccines have been developed since the outbreak continued spreading. Similarly, multiple studies have indicated that administering vaccinations containing mRNA, adenovirus vectors, or inactivated viruses can generate neutralizing antibody responses in older persons. A study showed that CoronaVac is highly immunogenic in adults aged above 60. Neutralizing antibody responses observed in groups of individuals receiving two vaccines at doses of 3µg or 6µg had similar results. The seroconversion rate and Geometric Mean Titer (GMT) of the neutralizing antibody were low before the second dose in the study's initial phase. In this trial, the GMT range for subjects who got doses of 3 g and 6 g after the second dosage was 42.2 to 64.4, and the seroconversion rate was 95%.[37]

Nonetheless, many studies still do not include groups of individuals who are immunocompromised, such as patients receiving immunosuppressants, patients in immunodeficiency states, organ donor recipients, and patients with malignancies undergoing chemotherapy with cytotoxic agents. Patients with malignancy usually have 10-30 times higher mortality rate than normal individuals.[38]

Several studies have shown a decrease in the immune response to both mRNA vaccines and primary infection of COVID-19 in immunocompromised individuals. However, this may differ depending on the type of treatment received by the patient.[39-42] For example, B-cell depleting antibodies for patients with autoimmune disorders or patients with chronic lymphocytic leukemia are thought to reduce humoral immune responses and vaccination effect. However, patients receiving anti-TNF therapy are still able to receive the vaccine.[40, 41, 42] Donor recipients are known to show poor antibody responses to mRNA vaccines. Similar to those who have solid or hematological malignancies, patients in this situation typically have a drop in antibody responses following the first vaccine but an improvement following the second immunization.[39] In immunocompromised patients (kidney transplant and CLL patients), the third dose of homologous vaccine mRNA induced an average increase in SARS-Cov-2 anti-spike IgG levels.[43]

Factors Affecting Each Group of Responder

In our previous study, hypertension has been noted as a factor lowering vaccine titers even after two doses of inactivated vaccine. It also was known to elevate the risk of breakthrough infection.[44] The following study on mRNA vaccine boosters after two doses of inactivated vaccine is still underway.

According to other literatures, patients with the haemtological disease are less likely to respond to the SARS-CoV-2 virus vaccine. Agha *et al.* (2021) showed that 46% of patients with haematological malignancies did not respond after four weeks of the second dose of SARS-CoV-2 mRNA vaccine.[45] Moreover, in patients with chronic lymphocytic leukemia, only about 23% of patients experienced seroconversion. Another study compared the antibody response to the third dose of mRNA vaccine (Pfizer/Moderna) with AstraZeneca in

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patients taking rituximab. The factor that causes a reduced response is the lack of B lymphocytes following the administration of immunosuppressant such as rituximab.[46]

Age is also an influential factor. A study conducted on groups of individuals with an age range of 18 – 59 years had seroconversion results of 97% and GMT 44.1.[18] However, in patients over 60, seroconversion and neutralizing antibody were lower at the first dose. Another study also compared the immune response in the age group of 80 and above and the younger age group, after the first vaccination. Patients above 80 had less binding IgG or IgA than the younger age group. The age group over 80 years old had decreased levels of interferon- and interleukin-2 production by SARS-CoV-2 spike-specific T cells. However, elderly patients had larger levels of SARS-CoV-2 spike-specific memory B cells following the second dosage of the vaccination.[47] This signifies that a good immune response occurred in the age group under 60, and a poor one occurred in the younger group.

Regrettably, the factors affecting the effectiveness of this booster are not extensively discussed in our study. We also did not conduct any quantitative analysis due to the unavailability of the specific software. Nonetheless, we managed to depict how booster vaccines, both heterologous and homologous, promote a better immune response. Follow-up studies on the influential factors affecting boosters' effectiveness are beneficially required.

The Impact of Booster Vaccine on Omicron Infection

Numerous theories have been put out to explain how Omicron, which has a high transmission rate, might evade the booster shot. Neutralizing antibody titers for Omicron were lower than those for the original strain and the Delta variation by 7.1-fold and 3.6-fold, respectively.[48]

In contrast to two weeks of the second dosage, the mRNA booster vaccine caused a >40-fold loss in neutralizing capacity against the Omicron version, according to animal experiments.[49] Mice were given either a homologous booster with LNP-mRNA or a heterologous booster with Omicron LNP-mRNA as a booster injection after two doses of the mRNA vaccines. Two weeks following the booster injection, compared to the day before the booster, the antibody response to Omicron jumped 40-fold. The heterologous Omicron LNP-mRNA booster evoked neutralizing titers 10–20 fold higher with equal titers against the Omicron variation compared to the homologous booster against that variation.[49]

According to Wi Ying Au and colleagues' investigation, COVID-19 infections brought on by the omicron variant can be successfully reduced by both homologous and heterologous three-dose regimens. Heterologous booster provides superior protection compared to homologous on Omicron.[50] The majority of recipients of three doses of CoronaVac did not produce neutralizing antibody responses to Omicron, according to just a research by Samuel M. S. Cheng et al.[51]

STRENGTH AND LIMITATION

The strength of this study was that this is the first literature discussing the administration of two COVID-19 vaccines with different platforms with a comprehensive manner based on previous human and animal trials in various countries. Whereas this study came out with a limitation to be conducted at the time when no trial on the combined effectiveness of two COVID-19 vaccine platforms was available, therefore, a quantitative study could not be performed.

CONCLUSIONS

As this pandemic still causes a continuous health burden, the vaccine has been one of

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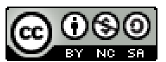

the worldwide significant steps in overcoming it. After two doses of vaccination, a booster vaccine with heterologous with mRNA-based is thought to improve the cellular and humoral immune systems, enhancing RBD antibody, NAb, and spike-specific Th1-type T cell responses.

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CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

AUTHOR CONTRIBUTION

Writer, literature searcher, collecting data from literature: BAM, Conceptor and supervision: GS, review and supervision: LW and DP.

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