

## Original Research Report

### EFFECTS OF *Moringa oleifera* EXTRACT AS AN IMMUNOMODULATOR OF LYMPHOCYTE CELLS AND MACROPHAGES IN BALB/c MICE INFECTED WITH *Plasmodium berghei*

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#### ABSTRACT

Malaria is an infectious disease caused by protozoa of the genus *Plasmodium*. In Indonesia, this disease remains a health concern to be resolved. Due to its high prevalence in eastern Indonesia, it is challenging to eradicate this disease. As an herbal alternative for disease eradication, *Moringa oleifera* that contains various substances is believed to have an antimalarial activity. This study aimed to determine the effectiveness of *Moringa oleifera* leaf extract in increasing immune cells and eradicating parasites by using mice infected with *Plasmodium berghei*. The research was conducted in vivo on BALB/c strain mice (n=40) that were already infected with *Plasmodium berghei*. *Moringa oleifera* leaf extract at 25%, 50%, and 75% concentrations was administered orally every day to the mice, while a peripheral blood smear was performed to evaluate parasitemia levels and macrophage activation. A complete blood count was also performed after all tests on the mice were completed. Statistical analysis was performed using the one-way analysis of variance (ANOVA) test with  $\alpha=0.05$  and 95% confidence interval (CI). The results showed that the administration of *Moringa oleifera* leaf extract at 25%, 50%, and 75% concentrations caused varying degrees of parasitemia compared to the negative group ( $p<0.05$ ). The group that received the extract at 50% concentration differed significantly from the control groups regarding the number of activated macrophages. The results of the complete blood count indicated immunomodulatory effects through the presence of diverse immune cell types. In conclusion, *Moringa oleifera* leaf extract suppresses *Plasmodium berghei* infection and enhances immune cell stimulation.

**Keywords:** Immunomodulator; macrophage; malaria; lymphocyte; *Moringa oleifera*

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#### Highlights:

1. This study assessed the immunomodulatory potential of *Moringa oleifera*, which may serve as a natural source for antimalarial treatment.
2. *Moringa oleifera* extract can act as an immunomodulator due to its suppressive effect on *Plasmodium berghei* infection.

## INTRODUCTION

Indonesia is among the countries known for having a tropical climate. The tropical climate is like a double-edged knife, presenting both advantages and disadvantages. One of the benefits associated with a tropical environment is the consistent exposure to sunlight throughout the year. Unfortunately, it also entails disadvantages such as higher temperatures,

the absence of the winter season, and certain health concerns. The health concerns arising from this climatic condition encompass the transmission of diseases spread by insects. *Anopheles* mosquitoes are a kind of insect that has a high degree of adaptability and can thrive in tropical conditions (Yahya et al. 2020). These mosquitoes are capable of sustaining their own lives while simultaneously spreading malaria. The disease is a result of a

*Plasmodium sp.* infection. Malaria exemplifies a pathological condition that targets the red blood cells. Consequently, complications may arise, particularly in relation to the quantity of red blood cells (Gultom et al. 2019).

The life cycle of malaria is typically categorized into three distinct stages: pre-erythrocytic, erythrocytic, and transmission. Upon the act of blood-feeding by a female *Anopheles* mosquito, the sporozoites are released into the host's bloodstream through the mosquito's saliva (Gultom et al. 2019). This particular stage is the start of the malaria life cycle. Initially, the sporozoites will penetrate the liver cells and form merozoites during the pre-erythrocytic stage. Merozoites will begin infecting erythrocytes and forming schizogony following the lysis of liver cells. This stage is known as the erythrocytic stage (Gultom et al. 2019). Upon the lysis of red blood cells, merozoites are released and subsequently proceed to re-infect these cells. After several cycles, gametocytes will develop and migrate to the mosquito's body when the mosquitoes feed on human blood. This process is referred to as the transmission stage.

The process of gametocyte fertilization results in the formation of a zygote, which subsequently develops into an oocyst and sporozoites. In addition to its detrimental effects on red blood cells and liver cells, malaria has the capacity to clog small blood vessels, including those found in the brain. This condition may induce tissue hypoxia (Fitriany & Sabiq 2018, Lee et al. 2022). The lethality of malaria is evident from its life cycle, particularly when inadequate treatments are applied.

In recent decades, malaria has emerged as a major problem in Indonesia. At the endemic level, malaria has had a rather benign impact on the community, despite the high prevalence of cases. According to the 2021 data provided by the Ministry of Health of the Republic of Indonesia, the number of active malaria cases was approximately 94,610 (Ministry of Health of the Republic of Indonesia 2022). The provinces of Papua, West Papua, and East Nusa Tenggara had the highest distribution of reported cases. Numerous attempts have been made by researchers to address this issue. Wardani et al. (2020) explored the potential of using *ashitaba* (*Angelica keiskei* (Miq.) Koidz.) stem ethanol extract as an herbal medicine for malaria. However, the current prevalence of malaria cases remains high, indicating that prior research efforts have not effectively contributed to the eradication of malaria. Additionally, there is a concerning emergence of malaria drug resistance, necessitating the development of novel antimalarial medications (Ministry of Health of the Republic of Indonesia 2022).

*Moringa oleifera*, commonly known as the miracle plant, possesses the potential to treat numerous health problems. *Moringa oleifera* harbors a range of secondary metabolites, including saponins, tannins, terpenoids, vitamin C, and flavonoids. The flavonoid compounds may exist in various forms, such as chalcones, kaempferol, alkaloids, phenols, and quercetin (Rivai 2020). The flavonoid content present in *Moringa* leaves is abundant in antioxidants. The antimalarial effect of the antioxidants has been reported to occur through the inhibition of malaria pathogen proliferation and the enhancement of immune cells (Veronica et al. 2020). One of the notable benefits of *Moringa oleifera* is its widespread availability in both local markets and supermarkets. On the other hand, the primary emphasis of dihydroartemisinin-piperazine (DHP) as a gold standard therapeutic intervention is on its antimalarial activities rather than its role as an immunomodulator. Immunomodulatory agents have the capacity to increase immune cells, including T lymphocytes and other activated cells, thereby accelerating the healing process when combating pathogens (Veronica et al. 2020). Therefore, the purpose of this study was to examine the effect of *Moringa oleifera* leaf extract on parasitemia and immune cells, specifically macrophages and lymphocytes.

## MATERIALS AND METHODS

This was a quasi-experimental study conducted in the animal breeding and research laboratory of the Faculty of Medicine and Health Sciences, Universitas Warmadewa, Denpasar, Indonesia. The experimental samples were BALB/c strain mice, around 7 weeks old and weighing approximately 25 grams. According to Federer's formula, a total of 40 mice were divided into five groups, with each group consisting of eight mice (Arwati et al. 2021, Molek et al. 2023).

The first group (negative control) received only water. The second, third, and fourth groups received *Moringa oleifera* leaf extract at varying concentrations of 25%, 50%, and 75%, respectively. The fifth group (positive control) received dihydroartemisinin-piperazine at a dose of 187.2 mg/kg BW (Arwati et al. 2021). The dihydroartemisinin-piperazine was manufactured in Indonesia by PT Mersifarma TM (6°09'15.8"S 106°07'73.9"E), with the batch number MTA-124486296. The *Moringa oleifera* leaves were acquired from a local market in Denpasar (08°14'17"S 115°05'02"E) and identified according to their morphology.

*Moringa oleifera* is a shrub or tree with strong roots. The plant is characterized by its long lifespan, brittle

woody stems, upright growth, dirty white hue, thin skin, rough surface texture, and rare branching (Benyamin 2023). The morphology of *Moringa oleifera* leaves is characterized by an oval shape and blunt edges. The size of the leaves is rather small. A single stalk of *Moringa oleifera* consists of a compound of leaves, as shown in Figure 1. *Moringa oleifera* has yellowish-white flowers that bloom all year with a distinctive fragrance. *Moringa oleifera* also bears long and triangular fruits with a length of about 20–60 cm. The fruits are typically green throughout their early stages of growth, and they turn brown as they ripen (Pratiwi 2018).



Figure 1. Morphological appearance of the *Moringa oleifera* stalk.

The preparation of *Moringa oleifera* leaf extract was performed by soaking refined dried leaves in 70% alcohol for three days. Afterward, the substance was filtered and evaporated to obtain a concentrated extract (Azzahra & Hayati 2019). The *Moringa oleifera* leaf extract was then diluted using distilled water in the appropriate ratios to achieve the desired extract concentrations of 25%, 50%, and 75%. Various concentrations of *Moringa oleifera* leaf extract were utilized as independent variables in this study. Prior to the experiment, all mice underwent a seven-day acclimatization. After the acclimatization period, blood containing 0.2 mL of the *Plasmodium berghei* parasite was injected into the peritoneal cavity of the mice (Farizal et al. 2020). The intraperitoneal injection was administered at an angle of 100 degrees relative to the abdomen, with a small inclination towards the midline. The procedure was performed with a cautious approach and at a moderate elevation to prevent any injury to the bladder or liver (Samson et al. 2019, Refdanita et al. 2020). The independent variables were administered orally to the mice starting on day 0 (two hours after infection) until day 4. The dosage protocol included the administration of 0.5 mL of *Moringa oleifera* leaf extract per 25 g of body weight in mice, with a frequency of once daily or a 24-hour time interval (Mustofa et al. 2019, Arwati et al. 2021). On day 5, only observation was conducted, with no further administration of the independent variables. The observation continued until day 6, which marked the seventh day of infection (Mustofa et al. 2019).

The results of administering the independent variables were observed on a daily basis during the experiment from day 1 to day 6. Consequently, six data sets were obtained for each independent variable (Kweyamba et al. 2019). Blood samples were taken from the tails of the experimental mice every day prior to treatment from day 1 to day 4, as well as 24 hours after treatment for days 5 and 6. The macrophage count was examined using peripheral blood smears and observed under a microscope following the Giemsa staining. In addition, a complete blood count was carried out to analyze the lymphocyte cells (Taek 2018, Hermanto et al. 2022). The macrophage morphology was observed in five fields of view. The number of infected erythrocytes was determined by assessing the degree of parasitemia across five fields of view and dividing by 1,000. This analysis showed whether the results represented normal erythrocytes in the five fields of view (Mustofa et al. 2019, Arwati et al. 2021, Juniantara et al. 2022).

All data obtained were analyzed using a one-way analysis of variance (ANOVA) test with a 95% confidence interval (CI) and  $p < 0.05$ . The statistical analysis was conducted using IBM SPSS Statistics for Windows, version 27.0 (IBM Corp., Armonk, N.Y., USA) (Mishra et al. 2019). Ethical approval for this research was obtained from the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, Universitas Warmadewa, Denpasar, Indonesia, with registration No. 320/Unwar/FKIK /EC-KEPK/IV/2023 dated 3/4/2023.

## RESULTS

### Degree of parasitemia in all groups

During the experiment in this study, it was observed that each group had various degrees of parasitemia with different patterns of fluctuations (Table 1). The mean degree of parasitemia in Group 1, which served as the negative control, was determined to be 14.68% on day 1. There was an increase seen on day 2, with a recorded mean degree of 16.67%. This trend continued until the last day, when it reached around 34.60%.

In this study, Group 2 received *Moringa oleifera* leaf extract at a concentration of 25%. On day 1, the mean degree of parasitemia was recorded as 7.77%, which subsequently climbed to 15.7% on day 2. However, on day 6, this group exhibited a mean degree of parasitemia of 10.2%, indicating a decrease compared to the previous day.

In the experiment involving Group 3, *Moringa oleifera* leaf extract was administered at a 50% concentration. On day 1, the mean degree of

parasitemia observed in this group was 7.34%, which subsequently increased to 13% on day 2. Nevertheless, on day 6, the group showed a reduction in the degree of parasitemia, which decreased to 7.29%.

Group 4 received *Moringa oleifera* leaf extract at a 75% concentration. The degree of parasitemia in this group exhibited a reduction from 8.6% on day 1 to 7.16% on day 2. The observed group exhibited an escalation in the degree of parasitemia until they recorded a decline to 9.6% on day 6.

Group 5, which served as the positive control, received dihydroartemisinin-piperazine at a dose of 187.2 mg/kg BW. The group exhibited a progressive decline in the degree of parasitemia. The mean value of the degree on day 1 was recorded as 6%, and it continued to decrease to 2% on day 6.

The results of the one-way ANOVA test indicated that the administration of *Moringa* leaf extract to mice infected with *Plasmodium berghei* led to a statistically significant reduction in the degree of parasitemia ( $p < 0.05$ ). Differences in the degree of parasitemia were seen between the positive control group and the groups given *Moringa oleifera* leaf extract at concentrations of 50% and 75%. However, the observed differences were statistically significant only until day 4.

Table 1. Degree of parasitemia observed in each group on a daily basis.

Day	Mean (%) degree of parasitemia					p
	Group 1	Group 2	Group 3	Group 4	Group 5	
1	14.68	7.77	7.34	8.60	6.23	0.00*
2	16.76	15.70	13.00	7.16	8.00	0.00*
3	22.73	15.30	13.01	13.16	6.55	0.00*
4	24.30	14.40	11.34	11.90	6.00	0.00*
5	25.73	10.40	8.61	11.23	4.30	0.00*
6	34.60	10.20	7.29	9.60	3.75	0.00*

Note: Significance level at  $p < 0.05$ .\*

### Activated macrophages in all groups

The observation revealed that the process of macrophage activation had occurred (Table 2). In Group 1, activated macrophages were obtained with a mean value of 2.83 on day 1. On day 2, there was a decline in the number, resulting in a mean value of 1.80. Subsequently, it reached a mean value of 2.2 on the final day.

Group 2 exhibited the activation of macrophages on day 1, with an observed mean value of 3.86. The value showed a rise to 4.5 on day 2. On the final day of the experiment, the group demonstrated a statistically significant rise in the mean value of activated macrophages.

On day 1, it was found that the mean activated macrophages in Group 3 were 6.13. On day 2, the measurement of activated macrophages revealed a reduction in the mean value to 6. On the last day of the experiment, there was a decline in the mean value of activated macrophages to 5.

The macrophages that were activated in Group 4 had an approximate value of 7.38 on day 1. The value subsequently declined to 4.29 on day 4. The activated macrophages in Group 4 exhibited a gradual decrease until day 4, followed by an upward trend that peaked at a mean value of 6.43 on the final day of the experiment.

In comparison to the treatment groups, it was observed that Group 5 had a mean activated macrophage value of 5 on day 1. The observed changes in the activated macrophages of this group were rather consistent. On day 2, there was a rise in the mean value to 6.33. On the final day of the experiment, the mean value of activated macrophages reached 6.5. According to the results of the one-way ANOVA test, the administration of *Moringa oleifera* leaf extract to mice that were infected with malaria resulted in a statistically significant increase in the number of activated macrophages observed from day 1 to day 4.

Table 2. Activated macrophages observed in each group on a daily basis.

Day	Mean** activated macrophages					p
	Group 1	Group 2	Group 3	Group 4	Group 5	
1	2.83	3.86	6.13	7.38	5.00	0.00*
2	1.80	4.50	6.00	4.29	6.33	0.00*
3	2.00	5.00	4.29	3.88	5.00	0.00*
4	2.50	3.33	5.13	2.75	4.33	0.01*
5	2.00	4.50	5.00	4.14	5.67	0.15
6	2.20	5.00	5.00	6.43	6.50	0.17

Notes: Significance level at  $p < 0.05$ \*; total macrophages in five fields of view\*\*.

### Complete blood count in all groups

According to the complete blood count of all groups, there was no statistically significant difference in lymphocyte cells ( $p = 0.63$ ) (Table 3). Nevertheless, when considering the average value, Group 3 exhibited the highest average. In the analysis of basophil cells, a statistically significant increase ( $p = 0.02$ ) was observed, with Group 3 having the highest number. Furthermore, the presence of healthy red blood cells suggested that the blood cells were not affected by *Plasmodium sp.* infection ( $p = 0.04$ ). Among the groups studied, Group 5 achieved the highest score, followed by Group 4 and Group 3.



Table 3. Results of the complete blood count.

Immune cells	Complete blood count (cell/mm <sup>3</sup> )					
	G1	G2	G3	G4	G5	p
Neutrophil	1.30	2.94	0.18	0.33	0.38	0.13
Lymphocyte	3.24	3.84	5.66	4.56	2.15	0.63
Eosinophil	0.11	0.03	0.04	0.03	0.00	0.43
Basophil	0.86	2.56	0.56	0.93	2.15	0.02*
Erythrocyte	5.54	6.77	6.95	7.56	7.86	0.04*

Note: Significance level at  $p < 0.05^*$ . G=Group.

## DISCUSSION

### Degree of parasitemia in all groups

This study revealed significant improvements throughout all observation days in the treatment groups administered with *Moringa oleifera* leaf extract. This might be due to the presence of many secondary metabolites within *Moringa oleifera* leaves (Rivai 2020, Islam et al. 2021). Flavonoids in *Moringa oleifera* leaves act as antioxidants, which can inhibit the growth of malaria pathogens and stimulate immune cells. A new permeation pathway can inhibit the process of membrane formation on Plasmodium, thereby preventing the degradation of hemoglobin (Marhaeni 2021). Consequently, *Plasmodium sp.* will be deprived of a vital nutrient source, leading to an inability to develop further. This is also important to prevent anemia, which usually occurs in malaria cases. Anemia has a tendency to progress into more severe illnesses, such as blackwater fever (Veronica et al. 2020).

Kaempferol in *Moringa oleifera* leaves has been found to have significant inhibitory effects on the production of reactive oxidative stress (ROS) and the process of fat peroxidation. These processes are known to contribute to the damage of cell membranes in malaria cases. Furthermore, *Moringa oleifera* leaves contain vitamin C, a compound that can enhance the enzymatic breakdown of hemoglobin (Hb) into ferrous iron (Fe<sup>2+</sup>). It is crucial that this action occur before malaria parasites start the degradation of infected hemoglobin. If the infected hemoglobin is damaged, it will result in the generation of ferric iron (Fe<sup>3+</sup>) and stimulate the release of electrons. Consequently, reactive oxidative intermediates (ROI) will be formed, leading to the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and damage to the host body. Vitamin C can also slow down the process of hemolysis and combat toxic cells as it possesses antioxidant properties (Veronica et al. 2020).

There was a statistically significant difference ( $p < 0.05$ ) in the degree of parasitemia between the negative control group and all other groups throughout all observation days. The findings of this study indicated that *Moringa oleifera* leaf extract had antimalarial activity. In contrast to the

positive control group, the administration of the extract at a 25% concentration showed a significant effect after day 2. The administration of the extract revealed significance starting from day 2 until day 4 at 50% concentration and from day 1 through day 6 at 75% concentration. The decrease in parasitemia observed in the treatment groups could not resemble the pattern in the positive control group, despite the presence of antimalarial activity. The findings suggest that the efficacy of *Moringa oleifera* leaf extract as an antimalarial medication falls short in comparison to dihydroartemisinin-piperazine. However, it is important to emphasize that the treatment group receiving *Moringa oleifera* leaf extract at 50% concentration showed a similar effect on the degree of parasitemia when compared to the positive control group. The provided evidence can serve as a starting point for future research projects (Mustofa et al. 2019, Obediah & Christian Obi 2020).

### Activated macrophage in all groups

The difference in the activated macrophages was only significant until day 4, particularly in the group receiving *Moringa oleifera* leaf extract at 50% concentration compared to the negative control group. *Moringa oleifera* is abundant in components that can stimulate interleukin-2 (IL-2) activity. As IL-2 stimulates lymphocyte cell proliferation, this substance is crucial after an antigen-presenting cell has presented *Plasmodium sp.* antigen to lymphocyte cells. After proliferating, lymphocyte cells will differentiate into T helper 1 (Th1) cells, which will activate macrophages after interferon gamma (IFN- $\gamma$ ) production (Widiani et al. 2021). Additionally, interferon gamma stimulates the transformation of monocytes into macrophages. As innate immune cells, macrophages will respond first to phagocytose pathogens (Mustofa et al. 2019, Subryana et al. 2020).

### Complete blood count in all groups

An immunomodulator refers to a compound that possesses the ability to improve the performance of the immune system. The immune system consists of a variety of cells, including neutrophils, basophils, eosinophils, and lymphocytes (Perdana 2021). Lymphocyte cells are primarily involved in the immune response against pathogenic microorganisms, the elimination of tumor cells or malignancies, and the prevention of organ transplant rejection. There are various factors that can contribute to an increase in lymphocyte cell count. Lymphocyte cells have several types, such as T helper lymphocyte cells, cytotoxic T lymphocyte cells, and B lymphocyte cells (Amran & Al Qarni 2019, Erniati & Ezraneti 2020).

T helper cells play a more significant role in activating other immune cells, including neutrophils, eosinophils, basophils, and B lymphocyte cells. By increasing basophils, T helper 2 (Th2) and T helper 9 (Th9) cells specifically combat parasitic infections such as malaria. Cytotoxic T cells destroy cells infected by pathogens. B lymphocyte cells produce antibodies that combat pathogens. In essence, lymphocyte cells have a broad spectrum of functions, so the role of lymphocyte cells in the immune response to malaria infection could not be specified in this study. Due to the inability of a complete blood count to determine the specific type of T helper, T cytotoxic, and B lymphocyte cells that had increased, additional research is required to analyze the count of lymphocyte cells according to their respective types (Prakoeswa 2020).

In this study, a significant increase in basophils was observed in the complete blood count. Basophils are cells that eliminate parasites after receiving information, specifically when interleukin-4 (IL-4) is produced by Th2 cells. Thus, an increase in basophils indirectly indicates an increase in Th2 cells (Tania 2020, Widiani et al. 2021). However, the complete blood count in this study also needs improvement. During the transport to the laboratory, the blood samples were placed in a cooler box, which caused several blood clots to form. Numerous cells were likely lost, affecting the results and causing discrepancies. There is a minimum quantity of blood that must be examined to perform a complete blood count, which makes it difficult to examine the blood of mice. Approximately 7.7 mL/kg BW was the maximum quantity of blood that could be extracted from mice. Since the mice in this study weighed between 25 and 30 grams, only about 1.9 mL of blood could be extracted before the mice died (Fatmawati et al. 2018).

This study did not assess potential adverse effects or toxicity. It is suggested that toxicity testing may be conducted in future research. The use of animal models in this study poses uncertainty regarding the generalizability of the observed effects to humans. However, human trials will proceed following the conclusion of this animal testing. In addition, *Plasmodium berghei*, a malaria species that infects rodents, is internationally recognized for its utility in studies of malaria remedies for humans. This is mostly due to its shared pathogenesis process with *Plasmodium falciparum*, which infects humans (Intan et al. 2020).

### Strength and limitations

The study provides information regarding the effects of *Moringa oleifera* leaf extract on both antimalarial activity and immunomodulatory

properties. Furthermore, this study can serve as a valuable resource for future research aiming to explore the potential of *Moringa oleifera* leaf extract as a therapeutic intervention for malaria and immunomodulation. However, it is important to acknowledge the limitations of this study, particularly in relation to the samples used for the complete blood count analysis. Several issues occurred during the study, including blood clots and frozen blood samples. Consequently, only a limited volume of blood samples was eligible to be analyzed. The findings from the complete blood count indicated the absence of cluster of differentiation 4 (CD4) among the total lymphocytes, which contributed to the severity of the malaria infection.

### CONCLUSION

*Moringa oleifera* leaf extract has antimalarial activity and immunomodulatory effects. However, it is not as effective as dihydroartemisinin-piperazine, which serves as the established standard medicine for malaria. Further research is required to explore varying intervals of dosage administration. The effectiveness of a complete blood count to assess lymphocyte count is limited due to the numerous lymphocyte types. Therefore, it is necessary to use a more precise method for assessing lymphocytes in future studies. It is also crucial to take the storing method of blood samples into account to preserve the quality of the samples.

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### Conflict of interest

None.

### Ethical consideration

This study obtained ethical approval for research feasibility from the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, Universitas Warmadewa, Denpasar, Indonesia, with registration No. 320/Unwar/FKIK/EC-KEPK/IV/2023 on 3/4/2023.

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#### Author contribution

PKDJ contributed to the conceptualization, study design, data collection, data analysis, data interpretation, methodology, manuscript writing, project administration, and content revision. PIBA contributed to the conceptualization, study design, data collection, data analysis, data interpretation, methodology, manuscript writing, content revision, supervision, and final approval. PMACPND contributed to the conceptualization, study design, data collection, methodology, and manuscript writing. DAASL contributed to the conceptualization, study design, content revision, supervision, and provision of samples. IKCAWS contributed technical and logistical support.

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