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# Inhibition of Interleukin-6 Expression by Curcumin in Rat Vascular Smooth Muscle Explants *In Vitro*

**[1]Yanti, [1]Stephanie, [1]Yuliani, [1]F.G. Winarno and [2]Maggy T. Suhartono**

*[1]Faculty of Biotechnology, Atma Jaya Catholic University, Jalan Jenderal Sudirman 51, Jakarta 12930, Indonesia*
*[2]Department of Food Science and Technology, Bogor Agricultural University, Bogor 16680, West Java, Indonesia*

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Corresponding Author:
Yanti,
Faculty of Biotechnology,
Atma Jaya Catholic University,
Jalan Jenderal Sudirman 51,
Jakarta 12930, Indonesia
Tel: +62 215703306 ext 722;
Fax: +62 21 5719060
Email: yanti@atmajaya.ac.id

**Abstract:** In atherosclerotic plaques, infectious agents from microbes may release Lipopolysaccharide (LPS) and heat shock proteins that can stimulate the production of mediators, i.e., pro-inflammatory cytokines, by vascular endothelial cells and Smooth Muscle Cells (SMCs). The elevated level of Interleukin-6 (IL-6) is strongly associated with the development of atherosclerosis. Here, we investigated whether curcumin isolated from the rhizome of *Curcuma longa* affected the expression of IL-6 at protein and gene levels in rat smooth muscle cells treated with LPS *in vitro* by conducting ELISA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assays. LPS at 2 μg $mL^{-1}$ activated the expression of IL-6 protein and mRNA in SMCs. Curcumin (1-15 μM) caused the decreased levels of IL-6 protein and mRNA in the dose-dependent manner in LPS-induced SMCs, indicating its potential antiatherosclerotic effect for cardiovascular risk management. In addition, curcumin also partially blocked the activation of LPS-induced phosphorylation of MAP kinases, i.e., ERK1/2, p38 and JNK, in SMCs, suggesting it may inhibit IL-6 expression via attenuating MAP kinase signaling pathways in LPS-induced SMCs. These data may in part explain the molecular action of antiatherosclerotic effects of curcumin.

**Keywords:** Curcumin, *Curcuma Xanthorrhiza* Roxb., Interleukin-6, MAP Kinase, Rat Smooth Muscle Cells

## Introduction

The use of medicinal plants for management of diseases is recognized to be associated with folk medicine from different parts of the world. Nowadays, natural products derived from plants have been well explored to be used in pharmaceutical purposes either as pure compounds or as extracts. For example, the rhizomes of *Curcuma longa* L. or turmeric, locally known as kunyit in Indonesia and belong to the Zingiberaceae family, have been widely studied due to its potential use for culinary and medicinal functions against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism and sinusitis (Araujo and Leon, 2001). *C. longa* rhizomes are rich in curcumin (diferuloylmethane), demethoxycurcumin, bisdemethoxycurcumin and essential oils (tumerone, atlantone and zingiberone). It has been noted that curcumin as the major constituent in *C. longa* exerted numerous pharmacological activities including anti-ulcer, anticancer, anti-inflammatory, anti-diabetic, anti-obesity and antimicrobial effects (Tuorkey and Karolin, 2009; Aggarwal 2010; Zhou *et al*., 2011). Due to its use as anti-inflammatory candidate, unfortunately, the efficacy study on inflammation-related atherosclerosis through modulating cytokine expression is rarely conducted till present.

Bacterial endotoxin or lipopolysaccharide promotes the activation of several kinases that are known to participate in inflammatory pathways including extracellular signal-related kinase 1/2 (ERK1/2) and p38 in vascular cells (Weinstein *et al*., 1992). The activation of these Mitogen Activated-Protein (MAP) kinase pathways is a critical mechanism by which LPS causes chronic inflammation and contributes to CVD. The three major groups of MAPKs that regulate gene expression are ERK1/2, p38 and c-Jun N-terminal Kinase (JNK). Mitogens and growth factors primarily activate ERK1/2. In contrast, JNK and p38 are activated by proinflammatory cytokines such as Interleukin (IL)-1β and Tumor Necrosis Factor (TNF)-α and cell stress-inducing factors such as heat shock,

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ultraviolet radiation and oxygen radicals (Han *et al*., 1993; Lee *et al*., 1994). In this study, we investigate whether curcumin isolated from the rhizome of *C. longa* might be effective for attenuating IL-6 expression in LPS-induced vascular smooth muscle cells via modulating MAP kinase signaling mechanism.

## Materials and Methods

### *Plant Materials*

Dried rhizomes of *Curcuma longa* Roxb. were collected from traditional markets in Jakarta and identified by Herbarium Bogoriense, Bogor Botanical Garden, Bogor. A voucher specimen (L003) is deposited in Faculty of Biotechnology, Atma Jaya Catholic University, Jakarta.

### *Isolation of Curcumin*

Curcumin was isolated from the methanol extract of *C. longa*. Briefly, the dried rhizomes (100 g) were grounded and extracted with 75% MeOH (v/v, 400 mL). The concentrated methanol extract was fractioned successively with ethyl acetate, n-butanol and water. Curcumin was isolated from the ethyl acetate fraction using silica gel column chromatography (Merck; 70-230 meshes, 5×43 cm, n-hexane-ethyl acetate 5 10:1, v/v). Curcumin was identified by direct comparison of the $^{1}$H-Nuclear Magnetic Resonance (NMR), $^{13}$C-NMR and Electron Ionization (EI)-mass spectral results with previously published data (Roughley and Whiting, 1973).

### *Cell Culture and Cell Viability*

Rat mesenteric artery smooth muscle cells were isolated and cultured as previously described by (Gunther *et al*., 1982) with slight modification. Briefly, male C-D strain Sprague-Dawley rats (225-250 g) were killed by cervical dislocation and the superior mesenteric arterial arcade was aseptically excised. Fat, adventitia and veins were removed by blunt dissection and the remaining arterial tree was incubated with elastase, collagenase, soybean trypsin inhibitor and bovine albumin at 37$^{o}$C in a gyratory shaker bath. After repeated tituration and sieving, the resulting cell suspension was washed by centrifugation and plated in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Serum Albumin (FBS), 2 mM L-glutamine, 25 mM HEPES buffer, 100 U/mL penicillin and 0.1 mg mL$^{-1}$ streptomycin. Similar methods were used for production of rat aortic smooth muscle cultures by enzymatic dissociation.

The cells were normally plated at a density of ~5×10$^{4}$ CFU/mL in plastic culture dishes or on glass coverslips that had been precoated with human fibronectin. Fibronectin-coated coverslips were used for increased cell adhesion; this treatment had no apparent effect on growth or staining patterns. Cultures were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ with thrice weekly media changes. The protocol has been approved by Animal Care and Use Committee of Primate Research Center, Bogor Agricultural University (Indonesia).

### *Sample Preparation*

Curcumin was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted to the final concentrations of 1-50 µM using 10% DMSO. Resveratrol as reference standard was also dissolved in 100% DMSO and diluted to the final concentration of 15 µM in 10% DMSO.

### *Enzyme-Linked Immunosorbent Assay (ELISA)*

IL-6 concentration in media were synthesized during inflammation was quantified by ELISA according to ELISA manufacturer's protocol (R&D Systems). Briefly, a 96-well plate that was pre-coated with a monoclonal antibody specific for rat IL-6 was incubated with 50 µL sample which has been diluted by calibrator diluents and standard or control to each well, following incubation for 2 h at room temperature. To wash specific unattached sample with antibody, plate was washed by wash buffer five times using automated plate washer. After the last wash, the plate was reversed in the paper towels to dry the wells for optimal results.

A 100 µL Horseradish Peroxidase (HRP) conjugated secondary antibody was added to wells and were incubated for another 2 h at room temperature and then washed five times. Plate was incubated 30 min with 100 µL substrate tetramethylbenzidine but protected from light exposure. The color will turn from blue to yellow was occurred after acidification with 100 µL HCl. During the procedures, the plate should be sealed with adhesive strip. OD was measured at wavelength of 450 nm by Microplate Reader with reference wavelength 595 nm using ELISA reader.

### *Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

Total RNA from LPS-induced artery SMCs was extracted using Trizol reagent according to the manufacturer's protocol and quantified spectrophotometrically at 260/280 nm. The cDNA was synthesized by using 1 µg total RNA, oligo(dT) primers and SuperScript III First-Strand (Invitrogen) in a 10 µL reaction. Reverse transcription was started at 50°C for 50 min and terminated at 85°C for 5 min. The cDNA products were diluted with sterile water up to 100 µL and PCR was conducted using 5 µL cDNA in a 25 µL reaction system with 0.5 U Takara LA Tag (Takara Bio).

The rat oligonucleotide primers for IL-6 and beta-actin were designed according to a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database. IL-6 primers were set up as 5' GGGTCGGTTCTGACCTTTTG3' for forward and 5' CTCTGTCCCTAATGCCCAGT3' for reverse.

Meanwhile, beta-actin primers were 5' AGCCATGTACGTAGCCATCC3' for forward and 5' CTCTCAGCTGTGGTGGTGAA 3' for reverse. PCR consisted of 30 amplification cycles and each cycle was carried out for 30 s at 94°C, 1 min at 55°C and 1 min at 72°C in a thermal cycler (Gene Amp PCR System 2700). The rat beta-actin housekeeping gene was used as an internal control to standardize the relative expression levels for MMP-9. PCR products were separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide. The stained gel was visualized by Gel-Doc Quantity One software (Bio-Rad). The relative expression level of IL-6 mRNA from the untreated and treated cells were calculated and expressed as ratios.

### *Western Blot*

To determine the expression of phosphorylated and total MAPKs, cellular lysates from the negative control, positive control and treatment group (curcumin) were prepared and assayed by Western blot analysis. Proteins (25 μg) were resolved by 10% SDS-PAGE and transferred to nitrocellulose transfer membranes. The membranes were blocked with 5% skim milk for 1 h at room temperature and then probed with the primary anti-mouse monoclonal antibodies against p-ERK1/2, p-p38, p-JNK at a 1:1000 dilution overnight at 4°C. After three washes, the blots were subsequently incubated with the secondary antibody peroxidase-conjugated anti-mouse IgG at a 1:4000 dilution for 2 h at room temperature. The blots were stained with SuperSignal West Femto Maximum sensitivity substrate (Thermo Scientific) and visualized using a LAS 3000 Bio Imaging Analysis System (Lab Science).

### *Statistical Analysis*

Triplicate experiments were performed throughout this study. All data are presented as the mean ± Standard Deviation (SD). The significant difference between control and treated groups were statistically analyzed by the paired Student's *t*-test ($p<0.05$).

## Results and Discussion

### *Effect of Curcumin on the Viability and Morphology of Vascular Smooth Muscle Cells in Vitro*

Curcumin (Fig. 1a), a hydrophobic phenol compound with molecular weight of 368, was successfully isolated from *C. longa* rhizomes. Curcumin was preliminary tested for its cytotoxicity against vascular smooth muscle cell viability and morphology. Our MTT results revealed that curcumin up to 15 μM did not affect both cell viability and morphology (Figs. 1b and 1c), suggesting the compound is not toxic within tolerance concentrations and used for further experiment.

### *Effect of Curcumin on the Expression of Interleukin-6 Protein and mRNA in Lipopolysaccharide-Induced Vascular Smooth Muscle Cells in Vitro*

As shown in Fig. 2, the presence of LPS significantly induced the expression of IL-6 protein level in the cell system. LPS plays a major role in stimulating the inflammation response including atherosclerosis and possesses various biological activities of pyrogenicity, mitogenicity, activated macrophages and adjuvant (Stimpson *et al*., 1987). Exogenous LPS causes the changes in metabolism and stimulates the production of pro-inflammatory cytokines (Interleukin (IL)-1, IL-6, Tumor Necrosis Factor (TNF)-α and Interferon (IFN)-γ) and inflammatory enzymes (MMP-9, Cyclooxygenase (COX)-2 and inducible Nitric Oxide Synthetase (iNOS) in various cells (Zhang *et al*., 2000). Our previous study showed that LPS stimulated the expression of MMP-2 and -9 in vascular epithelial cells *in vitro* (Yanti, 2010).

Further sample treatment using curcumin at various concentrations (5, 10 and 15 μM) was conducted. Our results demonstrated that curcumin dose-dependently caused the reduced level of IL-6 protein production in LPS-induced vascular smooth muscle cells *in vitro* (Fig. 2). Interestingly, it efficacy on decreasing IL-6 protein level was similar with the standard resveratrol. In line with this study, epigallocathecin gallate, areca nut, saikosaponin, peptidoglycan, curcuminoid and ginkgo biloba extract were also reported for their potential IL-6 inhibitory effects in various vascular and inflammatory cells (Schiborr *et al*., 2010; Wu *et al*., 2010; Lin *et al*., 2011; Lin *et al*., 2012).

At the gene level, curcumin also dose-dependently blocked the mRNA expression of IL-6 in the LPS-induced vascular smooth muscle cells *in vitro* (Fig. 3). These results clearly demonstrated that curcumin significantly affected the expression of IL-6 at protein and gene levels. Therefore, we further determined whether curcumin interfered signaling mechanisms mediated IL-6 gene expression in LPS-induced vascular smooth muscle cells *in vitro*.

### *Effect of Curcumin on MAP Kinase Signaling Involved in Interleukin-6 mRNA Expression in Lipopolysaccharide-Induced Vascular Smooth Muscle Cells in Vitro*

It has been known that LPS stimulate cytokine production including IL-6 through the activation of MAP kinases, nuclear factor-κβ, inhibitor κβ kinase and Activator Protein (AP)-1 in various cell culture and animal models (Cohen *et al*., 2009; Rafiee *et al*., 2009; Wang *et al*., 2009; Li *et al*., 2010). However, the upstream signaling molecules that responsible to regulate these signalings remain unclear in vascular smooth muscle cells.

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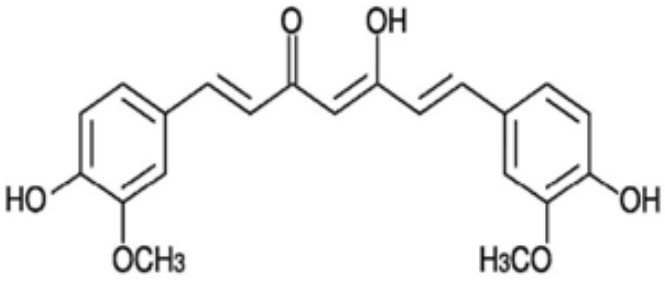
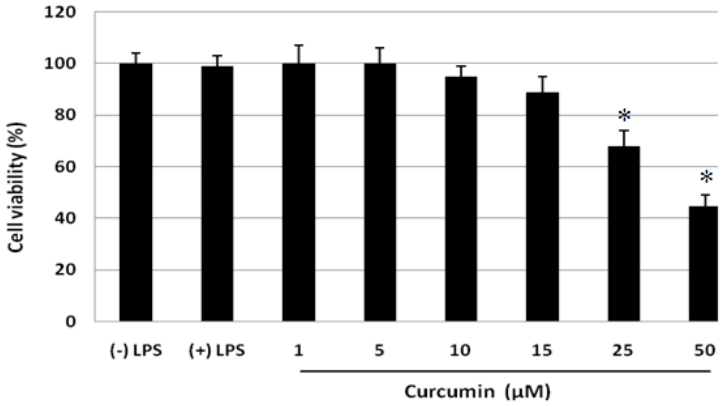



(a)

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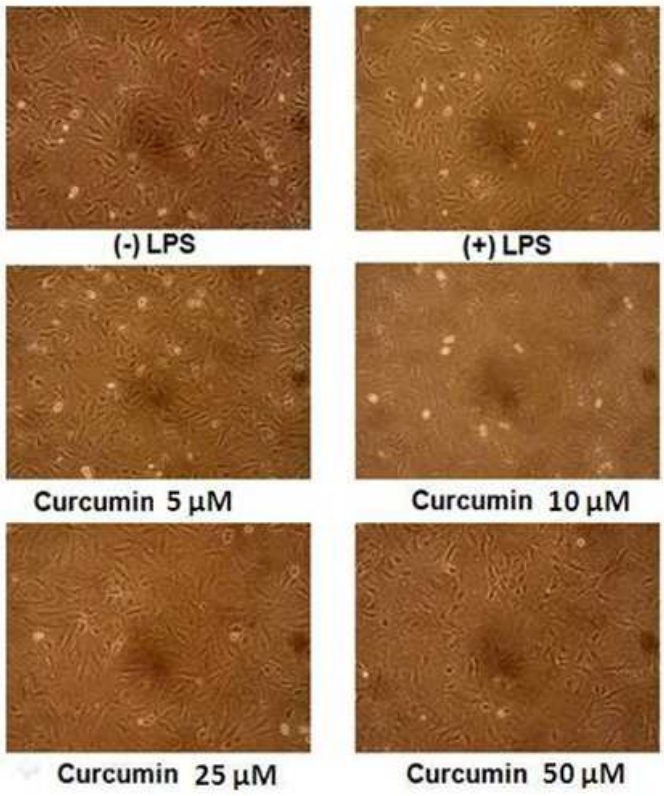


(c)

Fig. 1. Effect of curcumin (a) on vascular smooth muscle cell viability (b) and morphology (c) *in vitro,* **P*<0.05 vs LPS-treated cells

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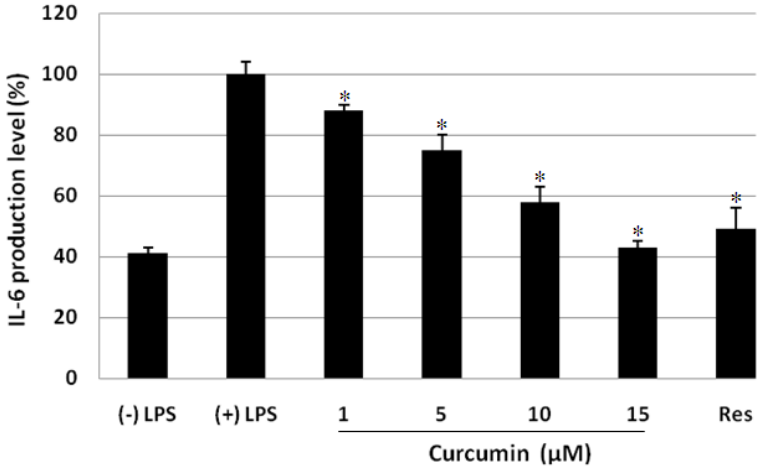


Fig. 2. Effect of curcumin on the level production of IL-6 protein in smooth muscle cells *in vitro* by ELISA assay, Res (15 μM) indicated resveratrol standard, **P*<0.05 vs LPS-treated cells

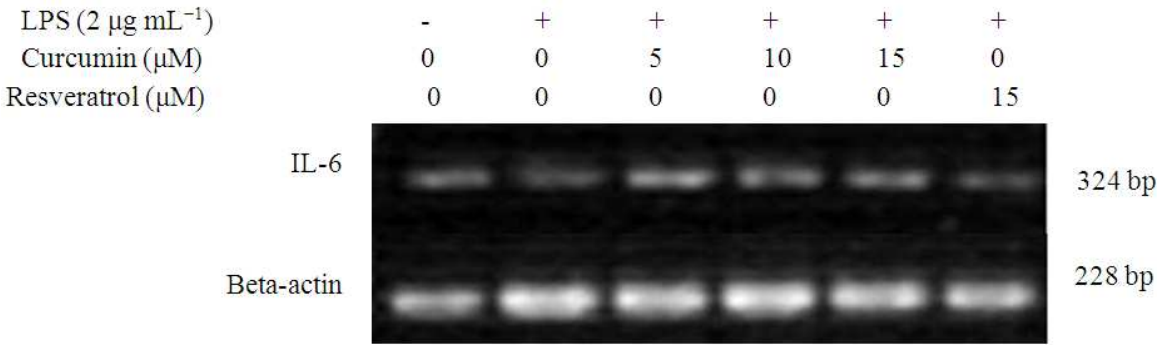


Fig. 3. Effect of curcumin on the expression of IL-6 mRNA in smooth muscle cells *in vitro* by RT-PCR assay. Res (15 μM) indicated resveratrol (standard)

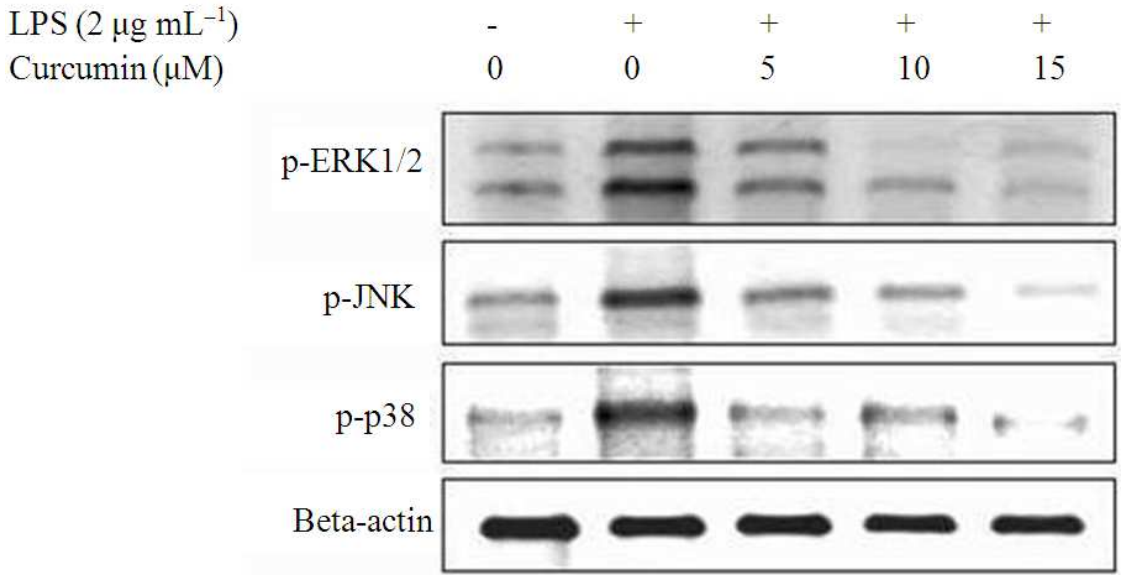


Fig. 4. Effect of curcumin on phosphorylation of ERK1/2, JNK and p38 in LPS-induced vascular smooth muscle cells *in vitro* by Western blot assay

In this study, LPS induced the expression of phosphorylated JNK, p38 and ERK1/2 in vascular smooth muscle cells (Fig. 4). Curcumin at concentrations of 5, 10 and 15 μM were found to equally inhibit the activating phosphorylation of JNK, p38 and ERK1/2 in the cell system. Our findings provide evidence for the role of curcumin on LPS-induced IL-6 gene expression by modulating MAP kinase signaling pathways in vascular smooth muscle cells. Together with this study, inhibition of MAPK phosphorylation by naturally-occurring agents presents an attractive strategy to block the excess production of IL-6 for prevention and treatment of CVD (Chang *et al*., 2009; Schiborr *et al*., 2010; Wu *et al*., 2010; Lin *et al*., 2011; Lin *et al*., 2012).

## Conclusion

The inhibition of phosphorylated JNK, p38 and ERK1/2 signalings may be one of the molecular mechanisms by which curcumin inhibits regulation of IL-6 mRNA expression in LPS-induced vascular smooth muscle cells *in vitro*. Taken together, it is worth to investigate whether curcumin exerts anti-atherosclerotic efficacy in animal model *in vivo*.

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## Author's Contributions

**Yanti:** Manuscript writing and result verification

**Stephanie:** Preparation for sample and cell culture

**Yuliani:** Preparation for sample treatment and molecular assays

**F.G. Winarno:** Data analysis, scientific discussion, and verification

**Maggy T. Suhartono:** Discussion, manuscript writing, and revision

## Ethics

All authors have read and approved the manuscript.

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