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Correlation between Salivary Lipopolysaccharide of *Porphyromonas gingivalis* with Circulatory Immunoglobulin-E and Immunoglobulin-G₄ in Periodontally Healthy Children with House Dust Mite Allergy

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Abstract

The presence of periodontal pathogens in periodontally healthy children is often overlooked or ignored. Since house dust mite allergy often appears among children with chronic gum disease, it is important to understand the role of lipopolysaccharide—a major immunodominant antigen of *Porphyromonas gingivalis*—in stimulating atopic inflammatory markers of allergies. The aim of the present study is to investigate whether any correlation exists between salivary lipopolysaccharide of *Porphyromonas gingivalis* with circulatory Immunoglobulin-E and Immunoglobulin-G₄ in periodontally healthy children with house dust mite allergy. We did an analytic observational study between January and December 2017. We recruited 20 periodontally healthy children (aged 6–16 years) from pediatric allergy-immunology clinic at Dr. Soetomo General Hospital (Indonesia). Lipopolysaccharide of *Porphyromonas gingivalis* was obtained from salivary secretion, while Immunoglobulin-E and Immunoglobulin-G₄ were obtained from venous puncture simultaneously. Immunoglobulin analyses were performed by direct-sandwich ELISA, and lipopolysaccharide analyses were performed by limulus amoebocyte lysate (LAL) assay. The average of salivary lipopolysaccharide was 7.21 ± 3.06 $\mu\text{g/ml}$, Immunoglobulin-E was 114.44 ± 26.19 pg/ml , and Immunoglobulin-G₄ was 31.02 ± 9.09 ng/ml . There was a strong correlation between salivary lipopolysaccharide and Immunoglobulin-E ($r^2 = 0.695$, $p < 0.001$), and a very strong correlation between salivary lipopolysaccharide and Immunoglobulin-G₄ ($r^2 = 0.796$, $p < 0.001$). *Conclusion*: data revealed significant correlations between salivary lipopolysaccharide of *Porphyromonas gingivalis* with circulatory

Immunoglobulin-E and Immunoglobulin-G₄ in periodontally healthy children with house dust mite allergy.

Keywords: Immunoglobulin-E, Immunoglobulin-G₄, lipopolysaccharide, periodontal pathogen, *Porphyromonas gingivalis*

1. Introduction

Given the complexity of community-based health care systems in the era of national insurance and high rate of poverty in Indonesia, it is not surprising that limited health literacy is common especially in rural areas [1]. Numerous studies show a link between limited parental health literacy with poor oral health among their child [2–4]. Parents' health literacy is often positively correlated with the frequency of dental visits and their knowledge/understanding of preventive measures in terms of oral or gingival diseases [5]. Thus, problems in the mouth range from bacterial biofilm, dental caries, chronic gingivitis, and locally aggressive gingivitis often reported in children whose parents have limited health literacy [6].

The presence of bacterial biofilm has an association with the presence of periodontal pathogen, which may lead to a periodontal disease when left untreated or undertreated [6]. Bacterial biofilm is often found among children with limited health literacy [7]. When left untreated or undertreated, these periodontal pathogens can spread below the gum line and may develop into gingivitis and periodontitis in the future [8]. Gingivitis is a reversible dental plaque-induced inflammation limited to the gingiva [9], while periodontitis is usually accompanied by gingivitis but involves irreversible destruction of the supporting tissues [10].

While many people believe that periodontal pathogen is an adult issue, nowadays this kind of pathogen is also a pediatric issue, since its presence is often reported in children aged 6–16 years [11, 12]. Even though the origin and transmission of these pathogens are not fully understood, few researches have linked them with salivary transmission. Saliva is the most probable vehicle for person-to-person transmission of periodontal pathogens, such as *Porphyromonas gingivalis* [13]. *Porphyromonas gingivalis*, a black-pigmented gram-negative anaerobic rod, is one of the most crucial periodontal pathogen, not only found in adults but also common in children [14]. It can be cultured occasionally from periodontally healthy mouths [14]. Their lipopolysaccharide and whole cell were detected in the gum and oral cavities of approximately 37% of people and at similar frequencies across ages and genders [15]. This fact highlights that *Porphyromonas gingivalis* may be acquired in the first days of life [16]. These results are intriguing, while some researchers argued that children may acquire these periodontal pathogens from their parents, especially if the parent has periodontitis [17].

Evidence suggests that the composition of the oral microbiome differs between children with and without allergy [18], and disruption of the bacterial biofilm in children leads to allergic responses following allergen challenge in subjects not previously sensitized to the allergen [19]. It is likely that the greatest concentration of *Porphyromonas gingivalis* lipopolysaccharides can be found in saliva, since it is a prerequisite for their transmission [20]. Hygiene hypothesis explained a protective role of microbiome (including oral microbiome) in the development of asthma and allergy [21]; on the other hand, in periodontally healthy and diseased mouths, *Porphyromonas gingivalis* in subgingival plaque elicit both local and systemic immune responses [22]. To our knowledge, however, studies investigating the direct

role of periodontal pathogens in terms of atopic inflammatory marker have not been reported.

The aim of the present study is to investigate whether any correlation exists between salivary lipopolysaccharide of *Porphyromonas gingivalis* with circulatory Immunoglobulin-E (Ig-E) and Ig-G₄. To accomplish this, we simultaneously measured salivary lipopolysaccharide, circulatory Ig-E, and Ig-G₄ in periodontally healthy children with house dust mite allergy.

2. Methods

2.1 Ethical considerations

This is the grant number for ethics approval (20/Panke.KKE/I/2017). A written informed consent was obtained from every parent to be included in the study. This study was approved by Institutional Review Boards of Dr. Soetomo General Hospital Ethics Committee for Health Research at Surabaya, January 20, 2017. Results will be disseminated through peer-reviewed publications within 1 year after experiment has been finished.

2.2 Research design and setting

This is an analytic observational study with cross-sectional study design to assess the correlation between salivary lipopolysaccharide of *Porphyromonas gingivalis* with the circulatory Ig-E and Ig-G₄. The study has been conducted by the support from Faculty of Dental Medicine Universitas Airlangga (Indonesia). Overall study started on January 1, 2017, and finished on December 31, 2017, at pediatric allergy-immunology clinic at Dr. Soetomo General Hospital (Indonesia) for data collection and Airlangga Oral and Dental Laboratory for IgE and IgG₄ measurement.

2.3 Sample size estimation

The sample size is based on a substantial meaningful change in IgE level observed in the group [23]. Korn et al. [23] stated that ELISA is able to assay for free IgE in a concentration range of 1–2000 pg/ml from peripheral blood samples with a substantial meaningful change of 0.5 pg/ml and the expected standard deviation of IgE concentration is assumed to be 0.01 pg/ml based on findings by Korn [23]. For this randomized controlled trial design, the formula is:

$$N = 2 \times \left(\frac{z_{1-\frac{\alpha}{2}} + z_{1-\beta}}{\delta} \right)^2 \times s^2$$

All parameters were assumed as follows: mean change of IgE in intervention group = 10 pg/ml; mean change of IgE in control group = 0 pg/ml; $\alpha = 0.05$; $\beta = 0.20$; $\delta = 10$ pg/ml; $s = 7$; and $s^2 = 49$.

We calculated and found the value of $N = 17.71$. This estimate requires 18 patients, to obtain 80% statistical power with 5% significance level for an independent samples and paired t-test. Due to the nature of this study being a pilot study, we aimed to recruit a minimal of 22 subjects in order to compensate for an estimate of 20% drop outs.

2.4 Participants

Study participants are recruited by direct invitation and pamphlets from pediatric allergy-immunology clinic at Dr. Soetomo General Hospital (Indonesia). Data collectors reviewed the clinic appointment schedules to identify subjects with HDM allergy and met with subjects and their parents or families after their physician visit for a screening to assess their eligibility. Patients were enrolled after considering various inclusion and exclusion criteria such as mentioned in (Table 1). By total sampling, 32 subjects were obtained from this hospital. They were prescreened from January 31 to February 28, 2017 at pediatric allergy-immunology clinic at Dr. Soetomo General Hospital (Indonesia). Based on the inclusion and exclusion criteria mentioned in (Table 1), 22 subjects were eligible to participate in the study. Informed consent is given as a voluntary agreement to participate in this research, out of which, 20 parents agreed and 2 parents declined to participate in the study.

2.5 Data collection

Data were collected by taking venous puncture for measuring total serum IgE and IgG₄ from subjects' blood samples and taking saliva for measuring lipopolysaccharide of *Porphyromonas gingivalis* concentrations [23]. Total serum IgE and IgG₄ were assessed by direct-sandwich ELISA (R&D System Europe Ltd., Abingdon, UK) according to the manufacturer's protocol. Briefly, total serum IgE was detected by diluting plasma (1:200), transferring it to pre-coated plates, and adding the supplied conjugate. However, total serum IgG₄ were detected using monoclonal antibody against IgG₄, followed by additions of blocking solution, diluted plasma sample (1:100,000) or standards, and conjugate, with washing between the steps. Total serum IgE and IgG₄ ELISAs were developed with the supplied TMB substrate and stop solutions. Total serum IgE and IgG₄ concentrations were determined using assay-specific 7-point calibration curves generated with the

Inclusion Criteria	Exclusion Criteria
Children aged 6–16 years at any gender and ethnic	Any sign of allergic diseases (asthma, hay fever, food allergy, eczema) within 1 month before observation
Have been diagnosed with HDM allergies by positive skin-prick test	Taking any antihistamines or steroid within 1 month before observation
Periodontally healthy children without any sign of chronic gingival diseases	Experienced dental scaling and root planning within 6 months before observation
Positive culture of <i>Porphyromonas gingivalis</i> from salivary samples in the absence of gingival diseases	The presence of low-grade fever due to infections rather than gingivitis/periodontitis
Understand and able to cooperate to the research protocol	Recent blood disorders or congenital abnormalities
Parents or legal representatives have signed the written consent in accordance with our institutional policies	Any medical conditions that may be harmful to be involved in this study, in which phlebotomy is contra-indicated

Table 1.
Eligibility criteria for study participants.

manufacturer-supplied standard. A value of 0.01 pg/ml was assigned for concentrations below the limit of detection [23].

We used a simple sample-processing method for PCR to detect *Porphyromonas gingivalis* from subjects' saliva [23]. We obtained 500 µL of bacterial culture from salivary secretion, added to 49.5 ml of sterile saline [23]. From this suspension, two serial 1:100 dilutions were made, and 0.1 ml was plated onto plate count agar from the last dilution [23]. Following that, lipopolysaccharides of *Porphyromonas gingivalis* were extracted by commercially optimized assay coupled with chromogenic substrate from pellet. We measured the concentration of extracted lipopolysaccharide by Pierce LAL Chromogenic Endotoxin Quantitation Kit. All measurements were done in duplicate and values averaged for analysis [23].

2.6 Statistical analysis

The obtained data were tabulated and analyzed using Statistical Package of Social Science (SPSS version 17, IBM, New York, USA). First, univariable linear regression procedures were conducted to examine associations between circulatory Ig-E and all determinants. In advance, dummy variables were created for all categorical determinants. Second, a multiple linear regression analysis with a stepwise exclusion method was conducted with all continuous and dummy variables. Determinants that seemed relevant for prediction of activation were kept in the model. Statistical significance was set at $p < 0.05$ [23].

3. Results

A total of 20 periodontally healthy children accepted to participate in this study; 8 were males and 12 were females. The average age was 10.52 ± 2.78 years (range, 6–16 years). The patients' general characteristics are as shown in **Table 2**.

3.1 Correlation between salivary LPS-Pg and Ig-E levels

Analysis of correlation between salivary LPS-Pg and Ig-E levels showed a strong correlation ($r^2 = 0.695$; $p < 0.001$, $n = 20$), and there was a positive correlation as shown in **Figure 1**.

3.2 Correlation between salivary LPS-Pg and Ig-G₄ levels

Analysis of correlation between salivary LPS-Pg and Ig-G₄ levels showed a very strong correlation ($r^2 = 0.796$; $p < 0.001$, $n = 20$), and there was a positive correlation as shown in **Figure 2**.

3.3 Results of univariate logistic regression analysis

As shown in **Table 3**, univariate logistic regression analysis uses gender, age, height, weight, BMI, family size, insurance status, number of colony-forming unit (CFU) of Pg, level of salivary LPS-Pg, level of circulatory Ig-G₄ as the independent variables, and circulatory Ig-E as the dependent variable. Data revealed that number of colony-forming units (CFU) of Pg, level of salivary LPS-Pg, and level of circulatory Ig-G₄ were associated with circulatory Ig-E ($p < 0.05$).

Variable	Mean (SD)	Minimum	Maximum
Gender (n)			
Male	8		
Female	12		
Age (years)	10.52 (2.78)	6	16
Height (cm)	140.28 (14.99)	108.50	162.00
Weight (kg)	36.12 (7.96)	23.00	51.00
BMI (kg/m ²)	18.24 (1.92)	15.27	21.82
Family size (n)	4.70 (1.38)	3	8
Insurance status (n)			
Public	17		
Private	2		
None	1		
CFU-Pg (×10 ⁵ CFU/ml)	9.93 (8.34)	1.08	29.45
LPS-Pg (µg/ml)	7.21 (3.06)	1.96	10.76
Ig-E (pg/ml)	114.44 (26.20)	77.69	141.41
Ig-G ₄ (ng/ml)	31.02 (9.09)	15.96	40.96

BMI, body mass index; CFU-Pg, number of colony-forming units of *Porphyromonas gingivalis*; LPS-Pg, level of salivary lipopolysaccharide of *Porphyromonas gingivalis*; Ig-G₄, level of circulatory Ig-G₄.

Table 2.
Demographic information of the study participants.

Figure 1.
Correlation between salivary lipopolysaccharide of *Porphyromonas gingivalis* concentration and circulatory Immunoglobulin-E level. Strong correlation between salivary lipopolysaccharide and circulatory Ig-E had been observed ($r^2 = 0.695$; $p < 0.001$, $n = 20$).

Figure 2.
Correlation between salivary lipopolysaccharide of Porphyromonas gingivalis concentration and circulatory Immunoglobulin-G₄ level. Very strong correlation between salivary lipopolysaccharide and Ig-G₄ had been observed ($r^2 = 0.796$; $p < 0.001$, $n = 20$).

Variable	β	S.E	p value	Min.	Max.
Gender	-0.130	7.953	0.418	-24.746	11.234
Age	0.360	2.904	0.273	-3.179	9.958
Height	-2.120	2.450	0.165	-9.248	1.838
Weight	1.814	4.858	0.250	-5.019	16.962
BMI	-0.950	9.665	0.212	-34.843	8.886
Family size	-0.181	2.501	0.202	-9.100	2.216
Insurance	0.155	6.252	0.247	-6.403	21.884
CFU-Pg	-0.082	0.466	0.595	-1.311	0.798
LPS-Pg	-0.017	2.271	0.951	-5.280	4.993
Ig-G4	0.999	0.758	0.004	1.163	4.595

BMI, body mass index; CFU-Pg, number of colony-forming units of Porphyromonas gingivalis; LPS-Pg, level of salivary lipopolysaccharide of Porphyromonas gingivalis; Ig-G₄, level of circulatory Ig-G₄.

Table 3.
The results of univariate linear regression analysis for Ig-E as dependent variable.

3.4 Results of multivariable regression analysis

Multivariable regression analysis was performed using indexes which were demonstrated to be related to circulatory Ig-E in univariate analysis. The results showed that only level of salivary LPS-Pg and level of circulatory Ig-G₄ remained a risk factor for circulatory Ig-E (Table 4).

Variable	β	S.E	Wald	P value	OR	95% CI
CFU-Pg	0.071	0.047	2.269	0.132	1.073	0.979–1.177
LPS-Pg	0.869	0.239	13.274	0.000	2.385	1.494–3.807
Ig-G4	0.196	0.068	8.271	0.004	1.271	1.064–1.390

CFU-Pg, number of colony-forming units of *Porphyromonas gingivalis*; LPS-Pg, level of salivary lipopolysaccharide of *Porphyromonas gingivalis*; Ig-G₄, level of circulatory Ig-G₄.

Table 4.
The results of multivariable logistic regression analysis for Ig-E as dependent variable.

4. Discussion

Despite existing literature pointing to a potential role of lipopolysaccharide in modulating allergic reactions, it remains a relatively underresearched subject matter. Notably, the majority of existing research has only investigated the interactions between periodontitis and clinical symptoms of allergies [24]. In this research, we had observed a direct correlation between lipopolysaccharide of *Porphyromonas gingivalis* with atopic inflammatory markers.

Lipopolysaccharide is a gram-negative endotoxin, ubiquitous in the environment and can therefore exacerbate allergic responses [25]. Existing literature has demonstrated the pathogenic role of *Porphyromonas gingivalis* dental biofilm to stimulate lipopolysaccharide-driven inflammatory responses [26], and therefore, lack of dental plaque and calculus in supra-gingival surface, sub-gingival surface, as well as human epithelial cell rests of Malassez account for the lack of response to lipopolysaccharide to induce host inflammatory responses [27].

As a unique endotoxin, lipopolysaccharide-driven inflammatory responses can induce a more pronounced pro-inflammatory cytokine response [28]. At very low concentration, lipopolysaccharide may induce atopic inflammatory responses by Th-1 shifting into Th-2, which is more potent to stimulate antibodies production [29]. Lipopolysaccharide binds to Toll-like receptor (TLR) 4 and greatly enhances the response of TLR4-transfected cells [30]. Thus, damage from lipopolysaccharide extends beyond the exhaustion of host innate immunity [31].

Lipopolysaccharide activates macrophage via the TLR4/NF- κ B pathway [32]. In turn, TLR4 activation increases SOCS3 mRNA expression [33]. Since SOCS3 is an inducible endogenous negative regulator of JAK/STAT pathway [34], therefore administration of lipopolysaccharide in a model of experimental periodontal disease will be correlated with dynamics of the atopic inflammatory reaction [35]. Meanwhile, given the unique lipopolysaccharide-induced atopic inflammatory responses and lipopolysaccharide-triggered mast cell derived, we can predict that lipopolysaccharide of this periodontal pathogen may stimulate the level of circulatory Ig-E and Ig-G₄, even in the healthy populations [36, 37].

In line with the hypothesis, our present study confirms the existence of a significant correlation between salivary lipopolysaccharide of *Porphyromonas gingivalis* with the circulatory Ig-E and Ig-G₄ in periodontally healthy children with house dust mite allergy. Importantly, this association remained even after adjusting for baseline and clinical parameters, suggesting an independent association between salivary lipopolysaccharide and allergic biomarkers.

This study had several limitations. Firstly, this study had small sample size, limiting the generalizability of the results. Perhaps more observations with longer periods would have been statistically significant with a larger sample size. Secondly, the cross-sectional approach of this current study cannot draw any

conclusions concerning direct relationships. Given the correlational nature of the analysis, we cannot clarify whether salivary lipopolysaccharide of *Porphyromonas gingivalis* is the direct cause of high circulatory Ig-E and Ig-G₄ in periodontally healthy children with house dust mite allergy. Lastly, this study did not assess the levels of house dust mite-specific serum Ig-E and the effect of increasing Ig-E level with the occurrence of allergic manifestation. Indeed, studies have recognized that evaluating an allergic manifestation and quantifying the levels of house dust mite-specific serum Ig-E are critical requisites when trying to establish an association with salivary lipopolysaccharide of *Porphyromonas gingivalis*. Nevertheless, this was beyond the scope of the present study, which aimed to investigate the correlation between salivary lipopolysaccharide and atopic inflammatory markers.

5. Conclusion

In conclusion, salivary lipopolysaccharide of *Porphyromonas gingivalis* might serve as a predictor for circulatory Ig-E and Ig-G₄ in periodontally healthy children with house dust mite allergy. These data might guide future studies on the actual role of these periodontal pathogens with the progression and sensitization of allergic diseases and help to establish a more effective treatment for child allergies. With increasingly more studies indicating the association of *Porphyromonas gingivalis* colonization and its lipopolysaccharide in an allergic child in the future, clinicians should be more aware about these periodontal pathogens in children's saliva and gum tissue. Despite a rare progression into a localized aggressive periodontitis, chronic gingivitis should be treated well in children, since its lipopolysaccharide may be linked with allergic diseases in the future.

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Conflict of interests

All authors declare no conflict of interests in this study.

Availability of data and materials

Most of the experimental data acquired/analyzed during this study have been included in this published version. Information on rest of the data is available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This is the grant number for ethics approval (20/Panke.KKE/I/2017). A written informed consent was obtained from every parent to be included in the study. This study was approved by Institutional Review Boards of Dr. Soetomo General Hospital Ethics Committee for Health Research at Surabaya, January 20, 2017. Informed consent was validated by the Institutional Review Boards of Dr. Soetomo General Hospital Ethics Committee for Health Research. All parents provided written informed consent to participate and were free to decline.

Author contributions

SCN designed the study and helped to draft the manuscript. RAN analyzed the data and initially wrote the manuscript. AE participated in the biochemical analysis. YDS participated in the preparation of the materials and carried out the experimental studies. HU participated in the figures preparation and analysis of data. SK, UT, and SP analyzed the data and critically revised the manuscript. All authors read and approved the final manuscript.

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