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Research Report

# Characterization of lactoferrin in gingival crevicular fluid of chronic periodontitis patient

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***ABSTRACT***

***Background:*** *Human periodontal diseases are inflammatory disorders as the result of complex interactions between periodontopathogens and the host's immune response. Periodontitis results in tooth loss and can even lead to systemic diseases if not treated. Gingival crevicular fluid (GCF) reflects the condition of the gingiva and contains proteins transuded from serum or cells at inflamated sites. Polymorphonuclear leukocyte (PMNs) infiltration can be seen in each stage of periodontitis. Lactoferrin is one of the PMN specific granules and could be a useful marker of PMN activity.* ***Purpose:*** *The aim of this study was to determine the band intensity of lactoferrin used as periodontitis biomarker.* ***Methods:*** *Gingival crevicular fluid (GCF) samples were collected using paper point no.30 from 40 subjects, 30 periodontitis patients that devide according to the severity (10 mild periodontitis, 10 moderate periodontitis, and 10 severe periodontitis) and 10 healthy controls, ranging in ages from 20 to 35 years. GCF lactoferrin was analyzed by Western blot and measured the band intensity by quantity one software (bio-rad).* ***Results:*** *The periodontitis sites exhibited significantly greater band intensity of lactoferrin than healthy sites. The band intensity of lactoferrin was positively correlated with the severity of periodontitis ($\alpha = 0.05$).* ***Conclusion:*** *The study showed that the intensity of the lactoferrin protein bands could be used as biomarkers of periodontitis.*

***Key words:*** *Gingival crevicular fluid, lactoferrin, periodontitis, band intensity*

***ABSTRAK***

***Latar belakang:*** *Penyakit periodontal adalah gangguan inflamasi yang merupakan hasil dari interaksi yang kompleks antara periodontopathogens dan respon imun host. Periodontitis mengakibatkan hilangnya gigi dan bahkan dapat menyebabkan penyakit sistemik jika tidak diobati. Cairan sulkus gingiva (GCF) mencerminkan kondisi gingiva dan mengandung protein yang tertransudasi dari serum atau sel pada lokasi radang. Infiltrasi polymorphonuclear leukosit (PMN) dapat dilihat pada setiap tahap periodontitis. Laktoferin adalah salah satu granula spesifik PMN dan bisa menjadi indicator aktivitas PMN.* ***Tujuan:*** *Tujuan penelitian ini adalah untuk meneliti intensitas band laktoferin dapat sebagai biomarker periodontitis.* ***Metode:*** *Cairan sulkus gingiva (GCF) dari tiap sampel dikumpulkan menggunakan paper pint no. 30 dari 40 subjek, 30 pasien dengan periodontitis yang dibagi sesuai dengan tingkat keparahan (10 periodontitis ringan, 10 periodontitis moderat, dan 10 periodontitis parah) dan 10 kontrol, mulai usia 20-35 tahun. GCF lactoferrin dianalisis dengan Western blot dan diukur intensitas bandnya dengan quantity one software (bio-rad).* ***Hasil:*** *Pada jaringan yang mengalami periodontitis menunjukkan intensitas band yang secara signifikan lebih besar dari laktoferin daripada periodontal yang sehat. Intensitas band laktoferin berkorelasi positif dengan tingkat keparahan periodontitis ($\alpha = 0,05$)* ***Simpulan:*** *Hasil penelitian ini menyimpulkan bahwa intensitas band protein laktoferin dapat digunakan sebagai biomarker periodontitis.*

***Kata kunci:*** *Cairan sulkus gingiva, laktoferin, periodontitis, intensitas band*

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## INTRODUCTION

Periodontitis is a periodontal disease due to the development of the inflammatory process in the gingival tissue that continues to the other support tissues.[1] Bacterial biofilms are regarded to be the primary aetiological factor in the initiation of gingival inflammation and subsequent destruction of periodontal tissues and three major specific pathogens have been repeatedly identified as etiologic agents, namely *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg) and *Tannerella forsythia* (Tf). Although chronic exposure to bacteria and their products is a prerequisite for gingival inflammation and periodontal tissue destruction to occur, the major causative factor of soft- and hard- tissue breakdown associated with periodontitis is currently attributed to the host's immune-inflammatory response to bacterial challenge. Furthermore, the nature of the inflammatory response might determine the destructive character of the disease.[2]

*Porphyromonas gingivalis* is one of the major pathogens in chronic periodontitis. *P. gingivalis* has a number of virulence factors such as capsule, fimbriae, lipopolysaccharide (LPS) and potent proteolytic enzymes such as gingipains. These factors can induce an inflammatory cascade involving pro-inflammatory cytokines, reactive oxygen species and matrix metalloproteinases (MMPs), thus leading to the destruction of supportive soft and hard tissues around the teeth.[3]

The movement of leukocytes from the blood to the local tissue is the key in process of inflammation. Trans-endothelial migration is a selective interaction between leukocytes and the endothelium resulting in leukocytes out of the bloodstream into the tissues. Local damage trigger a variety of inflammatory signals (IL-1 β, TNF-α), mainly derived from resident leukocytes such as mast cells. Mast cells are an important part which triggered the mobilization of PMN against bacteria and response to anaphylatoxins such as C3a and C5a.[1] PMN acts as a barrier between the mass of plaque and crevicular epithelium.[4]

A diagnosis of periodontitis is established by traditionally used indices, e.g., bleeding on probing and probingdepth, which indicate the loss of periodontal tissue attachment to the teeth. Additionally, radiographyvisualizes the loss of periodontal tissue, which supports the diagnosis by determining the amount of bone lossaround the teeth. However, these methods are only useful when attachment loss has occurred to somedegree. For chair-side and single visits, more reliable biomarkers for periodontitis are needed to provide constant classification and customized treatment and for monitoring periodontal diseases.[5]

Gingival crevicular fluid (GCF) is bodily fluid that reflects the condition of the periodontium, contains various components that originate by transudation of the serum or inflammatory factors and are derivedfrom the interaction between the bacterial biofilm and the cells of periodontal tissues. Additionally,despite a large variation, the amount of GCF tends to increase with the severity of gingival inflammation. In addition to these characterizations, the simple and noninvasive collection of GCF is required for the discovery of periodontitis biomarkers.[5,6]

Lactoferrin is a glycoprotein with a molecular weight of about 80 kDa, which shows high affinity for iron, and classified as a member of the transferrin family, due to its 60% sequence identity with serum transferrin.The ability to keep iron bound even at low pH is important, especially at sites of infection and inflammation where, due to the metabolic activity of bacteria, the pH may fall under 4.5. In such a situation lactoferrin also binds iron released from transferrin, which prevents its further usage for bacterial proliferation.[7]

Lactoferrin can be found in saliva and crevicular fluid and acts bacteriostatic and bactericid. In the crevicular fluid significant higher levels of lactoferrin have been found at periodontitis sites in comparison to healthy sites.[6] Lactoferrin is one of the PMN specific granules and could be a useful marker of PMN activity.Increased lactoferrin levels have been reported in severe infections, for example, meningitis, in burns patients, rheumatoid arthritis, and chronic salivary gland diseases. There are few reports about the quantification of lactoferrin in GCF. Lactoferrin of gingival crevicular fluid levels in gingivitis, periodontitis, and localized aggressive periodontitis patients were two-fold higher than in periodontally healthy individuals. Gingival crevicular fluid production is a result of the increase in permeability of microvasculatures of the periodontal tissue due to inflammatory reactions. Lactoferrin released from PMNs into the GCF is a good indicator of periodontal inflammation.[8] The objective of this study was to determine the band intensity of lactoferrin used as periodontitis biomarker.

## MATERIALS AND METHODS

GCF samples were collected from periodontitis patients and healthy individuals for the identification of periodontitis biomarkers at the Department of Periodontology, Faculty of Dental Medicine Universitas Airlangga. The standard protocol was approved by the KKEPK (Komisi Kelaikan Etik Penelitian Kesehatan) of Faculty of Dental Medicine Universitas Airlangga (KKEPK NO. 151/KKEPK.FKG/X/2013). Informed consent was obtained from all donors.

Forty male patients in range of age 20 to 45 years old participated in the study. All participants were free of systemic disease and had not taken medication (such as anti-inflammatory agents, antibiotics, immunosuppressants, or contraceptives) that could affect their periodontal status for at least six months prior to the study. Radiographic examination and clinical periodontal assessment were performed. To stratify chronic periodontitis patients, the clinical periodontal parameters from average 6 sites per individual were assessed at the initial examination for mean probing depth (PD), clinical attachment loss

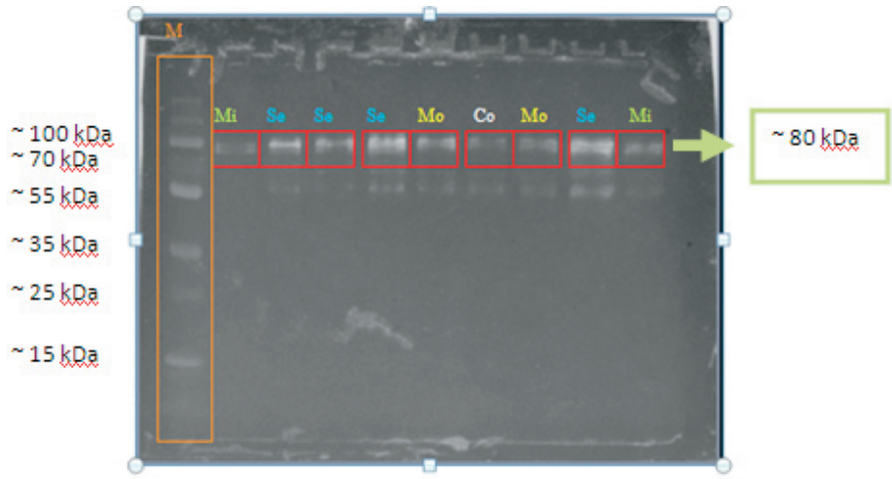


**Figure 1**. Westernblot result of lactoferrin; M = Marker; Co = kontrol; Mi = *mild*; Mo = *moderate*; Se = *severe.*

(CAL), and bleeding on probing (BOP). According tothe criteria, we divided the samples into 4 groups, healthy, mild periodontitis, moderate periodontitis, and severe periodontitis based on clinical attachment loss (CAL), healthy subjects with no CAL, mild periodontitis with 1-2 mm of CAL, moderate periodontitis with 3-4 mm of CAL, and severe periodontitis with more than 5 mm of CAL.

GCF was taken from the tooth at posterior region of maxilla with the deepest pocket. Each tooth was issolated with a cotton roll and the supragingival plaque was carefully removed without touching the marginal gingiva. The gingiva was then gently dried with an air syringe. Gingival crevicular fluid was collected using paper point No. 30 at the orifice of the sulcus 1-2 mm subgingivally for 30 seconds. The same procedure was repeated after 1 minute. The paper points werethen stored in 100 µL PBS (pH 7.4) and then stored at -40° C until further analysis

The entire sample were calibrated for their total protein concentration using a nano drop before SDS-PAGE analysis. SDS-PAGE analysis performed to separate proteins based on molecular weight. After SDS-PAGE analysis, proteins in the gel were then transferred onto nitrocellulose (NC) membranes. NC membranes were incubated in primary antibody (anti-lactoferrin antibody) at a ratio of 1: 1000 in 1% skim milk solution overnight, then washed with PBST. After the membranes were incubated in secondary antibody labeled with alkaline phosphatase which has a ratio of 1: 2500 for 1 hour, then washed with PBST. The membranes were incubated with Western blue substrate solution in the dark until visible color bands or overnight, then washed with distilled water. Then the membranes were dried at room temperature.

The membranes were documented, furthermore, each image is analyzed to calculate the intensity of bands with Quantity One software. Data analysis was performed using one-way ANOVA. The correlation among the intensity of lactoferrin band and the severity of chronic periodontitis were assessed using Tukey HSD test.

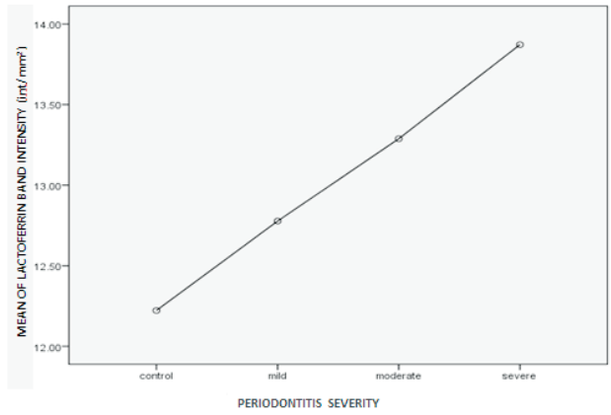


**Figure 2**. The increase of lactoferrin intensity.

**Table 1**. Lactoferrin bands intensity of gingival crevicular fluid from each severity of chronic periodontitis

| No | Control (int/mm$^2$) | Mild periodontitis (int/mm$^2$) | Moderate periodontitis (int/mm$^2$) | Severe periodontitis (int/mm$^2$) |
|---|---|---|---|---|
| $\overline{X}$ | 12,22* | 12,78 | 13,29 | 13,87 |
| SD | 0,25 | 0,17 | 0,2 | 0,18 |

*Data presented as mean ± SD

## RESULTS

Figure 1 showed that protein bands were thicker with a higher color intensity than others. The increase of lactoferrin intensity was shown in Figure 2.

The mean of the control group was higher than the group with mild periodontitis, moderate and severe, so it can be seen that there is an intensity increase at each level of periodontitis severity (Table 1).

## DISCUSSION

The role of bacteria in periodontal disease progression has been widely recognized, but in reality the innate immune systems were more responsible for damage to bones, and ligaments that support the teeth and gingiva. Initiation of bacteria causing PMN migrate to the site of infection. Polymorphonuclear leukocytes (PMN) penetrate the connective tissue into the gingival sulcus through intercellular junctional epithelium,[9] and are responsible for detecting and eliminating bacteria. Polymorphonuclear leukocytes loaded with enzymes that have a high toxicity called protease. This enzyme is also responsible for the damage caused during a prolonged inflammatory phase that occurs when PMN become hyperactive or chronically activated by bacterial stimulus.[10]Excessive host response may lead to tissue damage that occurs depends on the length of PMN in the tissue. The existence of PMN in the tissue for a long time can lead to tissue damage.[9]

During phase active of periodontal disease, cell death occurs and intracellular content are released. Cell death through apoptosis is essential for maintaining the function and integrity of the tissue. Apoptotic cells can help resolve inflammation by sending a signal to the macrophages to engulf PMN and secrete anti-inflammatory cytokines, such as TGF-β. If the PMN did not undergo apoptosis, the number and activity of macrophage will also decrease, so that PMN will continue to release the destructive contents. Thus, in addition to killing bacteria, PMN can continue to release products that can cause direct damage to the extracellular matrix and other host cells.[9] Cytokines such as TNF-α, monocytecolony granulocyte stimulating factor (GM-CSF) may cause a delay of PMN apoptosis by increasing the stability of its mitochondria, decrease the activity of caspase 3 and downregulated gene expression of Bax which is a pro-apoptotic member of the Bcl-2. Recent studies also indicate that the products of bacteria isolated from strains of Porphyromonas gingivalis can also delay PMN apoptosis.[11]

PMN is the largest source of lactoferrin in adults. Lactoferrin is mostly stored in the specific granules (secondary granules), but can also be found in tertiary granules although in lower concentrations.[7,8] PMN go to the site of infection in response to chemotactic molecules that are activated by bacteria, such as complement proteins, interleukin-1, and interleukin-8. Bacteria are recognized and engulfed by PMN into the phagosome. Phagosome membranes fused with granular cytoplasmic membrane, resulting in release of granule contents (degranulation) into the phagosome and the extracellular environment to kill bacteria.[9]

Lactoferrin is found in the saliva and crevicular fluid, and acts bacteriostatic and bakteriosid.[6] In this study, the presence of lactoferrin derived from the saliva have been eliminated at the time of sampling, so that lactoferrin were identified only from the GCF. Iron levels in GCF increased in a periodontal disease, iron can increase oxidative stress by catalyzing the formation of ROS through the fenton reaction and increase the growth of certain periodontopatogen, so that increased iron in GCF can be harmful to periodontal tissues. Iron is important for the survival of the bacteria. Lactoferrin binds to iron to prevent the progression of fenton reaction, so the increased levels of GCF lactoferrin in periodontitis may act as an antimicrobial agent and antioxidant preventive.[12]

Gingival crevicular fluid was taken from male patients with chronic periodontitis, do not have a systemic disorder and nonsmokers. The determination of these criteria due to hormonal influences. Sex differences have been reported to affect the body's response to bacteria.[13]Smoking is also associated with the etiology and pathogenesis of periodontal disease, as well as to the results of periodontal treatment.[14] Smoking also has a detrimental effect on the survival and function of PMN in GCF in populations with healthy periodontal.[15]

In this research, lactoferrin in gingival crevicular fluid was characterize using Western blot method. Previous research has shown a positive relationship between the titers of lactoferrin periodontitis in GCF. According to Kivadasannavar *et al*.,[16] a statistically significant difference exists between the LF levels before and after calling and root planning and periodontal flap surgery. A correlation has been found between the lactoferrin levels in GCF and the treatment modalities carried out. That result is in accordance with the investigation done by Jentsch *et al.*,[6] who conclude that there is a decrease in lactoferrin found in GCF of periodontitis patients during periodontal therapy. Another study conducted by Glimvall *et al.,*[7] concluded that higher levels of lactoferrin were detected in subjects with chronic periodontitis and correlated with probing pocket depth ≥ 6 mm. Although some data have been raised regarding the titers of lactoferrin in GCF of periodontitis patients, but this study is the first to reveal the relationship between the intensity of the band lactoferrin in the GCF of patients with periodontitis severity.

The band is formed because there is a specific reaction between antigen with antibody. Primary antibodies used in this study is a monoclonal antibody lactoferrin, so that antigen-specific binding protein lactoferrin. The specific proteins can be used for serological diagnosis by conducting further tests to obtain high specificity and sensitivity.[18]

The calculations using Quantity One show that there is an intensity increase of lactoferrin bands in accordance with the increase in periodontitis severity. In the control group appeared lactoferrin band with low intensity. This indicates that the gingival crevicular fluid of healthy periodontal tissues also contained lactoferrin in low concentrations, as in the human oral cavity also contained a constant amount of bacteria in a controlled. Continuous influx of PMN into the gingival pockets surrounding periodontal tissues play a major role in the control,[10] whereas in the group with periodontitis, lactoferrin band intensity thicken with

increasing severity of periodontitis. This is in accordance with the opinion of Moosani which states that the PMN was found in the pocket of clinically healthy gingival tissue, and showed an increase in the number of chronic inflammation.[9]

Anova showed a significant difference in lactoferrin band intensity. According Kusnoto, pure isolates and a good levels of protein homogenates will produce a good and clear protein bands. There is a dominant protein bands with bold colors and there is expressed by the form of a thin bands. This is due to the good level of purity and protein concentration were sufficient. This means that there are differences in the concentration of lactoferrin in any degree of periodontitis severity.[20]

Tukey test results indicate that there are significant differences between each group of samples. Severity increase of chronic periodontitis followed by an increase in the intensity of lactoferrin bands. The Increased of band intensity indicates an increase in the concentration of lactoferrin that contained in gingival crevicular fluid of each sample. This illustrates that the increase in the chronic periodontitis severity affects the concentration of lactoferrin in gingival crevicular fluid. These results are in accordance with the opinion of Adonogianaki *et al.*[14] and Ozdemir *et al.*[19] that there are different levels of lactoferrin in GCF significantly between patients with gingivitis and periodontitis compared with healthy subjects. Tsai *et al.*[8] also revealed that there is a strong relationship between periodontal clinical parameters with levels of lactoferrin. Increased severity of periodontal inflammation followed by an increase in GCF lactoferrin. Similar results have also been reported that myeloperoxidase derived from PMN associated with GCF volume, in addition to the β-glucorodinase also has a relationship with periodontal pocket depth. It may reflect a decrease in neutrophil chemotactic factor in the pocket due to the elimination of plaque and periodontal inflammation.[14]

GCF production is the result of an increase in microvascular permeability due to periodontal tissue inflammatory reaction. Lactoferrin that is released from PMN into the GCF could be a usefulmarker of periodontal inflammation. Therefore lactoferrin levels in GCF is a sensitive and objective method for detecting the severity of periodontitis.The results of this study showed that the intensity of the lactoferrin bands could be used as a biomarker of periodontitis. Further research needs to be directed towards the developing a chairside diagnostic kit to collect and evaluate the concentration of lactoferrin in the clinic.

## REFERENCES


1. Newman MG, Takei HH, Carranza FA, Klokkevold PR. Clinical periodontology. 10th ed. Philadelphia: Elsevier Saunders; 2006. p. 494.
2. Gemmell E, Yamazaki K, Seymour GJ. Destructive periodontitis lesions are determined by the nature of the lymphocytic response. Crit Rev Oral Biol Med 2002; 13(1): 17-34.
3. Kuula H, Salo T, Pirila E, Tuomainen AM, Jauhiainen M, Uitto VJ, Tjaderhane L, Pussinen PJ, Sorsa T. Local and systemic responses in matrix metalloproteinase 8-deficient mice during Porphyromonas gingivalis-induced periodontitis. Infect Immun 2009; 77(2): 850-9.
4. Wilson TG, Kornman KS. Fundamentals of periodontics. Slovakia: Quintessence Publishing Co. Inc; 2003. p. 160-6.
5. Choi YJ, Heo SH, Lee JM, Cho JY. Identification of azurocidin as a potential periodontitis biomarker by a proteomic analysis of gingival crevicular fluid. Proteome Science 2011; 9: 42.
6. Jentsch H, Sievert Y, Go¨cke R. Lactoferrin and other markers from gingival crevicular fluid and saliva before and after periodontal treatment. J Clin Periodontol 2004; (31): 511–4.
7. Adlerova L, Bartoskova A, Faldyna M. Lactoferrin: a review. Veterinarni Medicina 2008; 53(9): 57–468.
8. Tsai CC, Kao CC, Chen CC. Gingival crevicular fluid lactoferrin levels in adult periodontitis patients. Aust Dent J 1998; 43(1): 40-4.
9. Moosani A. Evaluation of oral neutrophil levels as a quantitative measure of periodontal inflammatory load in patients with special needs. Thesis. Toronto: Department of Paediatric Dentistry Faculty of Dentistry University of Toronto; 2012.
10. Glogauer M. Neutrophil research: the future of periodontal diagnosis and treatment. PEAK of Royal College Of Dental Surgeon Ontario; 2007. p. 1-8.
11. Gamonal J, Sanz M, O'Connor A, Acevedo A, Suarez I, Sanz A, Martı´nez B, Silva A. Delayed neutrophil apoptosis in chronic periodontitis patients. J Clin Periodontol 2003; 30(7): 616–23.
12. Wei PF, Ho KY, Ho YP, Wu YM, Yang YH, Tsai CC. The Investigation of Glutathione Peroxidase, Lactoferrin, Myeloperoxidase and Interleukin-1b in Gingival Crevicular Fluid: Implications for Oxidative Stress in Human Periodontal Diseases. J Periodont Res 2004; 39: 287–93.
13. Kowolik MJ, Dowsett SA, Rodriguez J, De La Rosa RM, Eckert GJ. Systemic neutrophil response resulting from dental plaque accumulation. J Periodontol 2001; 72(2): 146-51.
14. Ozdemir B, Ozcan G, Karaduman B, Teoman AI, Ayhan E, Ozer N, Us D. Lactoferrin in gingival crevicular fluid and peripheral blood during experimental gingivitis. Eur J Dent 2009; 3(1): 16-23.
15. Guntsch A, Erler M, Preshaw PM, Sigusch BW, Klinger G, Glockmann E. Effect of smoking on crevicular polymorphonuclear neutrophil function in periodontally healthy subjects. J Periodontal Res 2006; 41(3): 184-8.
16. Kivadasannavar AV, Pushpa SP, Sanjeevini AH, Soumya SK. Estimation of lactoferrin levels in gingival crevicular fluid before and after periodontal therapy in patient with chronic periodontitis. Contemp Clin Dent 2014; 5(1): 25-30.
17. Glimvall P, Wickstrom C, Jansson H. Elevated levels of salivary lactoferrin, a marker for chronic periodontitis?. J Periodont Res 2012; 47(5): 655-60.
18. Kresno SB. Imunologi: diagnosis dan prosedur laboratorium. Edisi kelima. Jakarta: Fakultas Kedokteran Universitas Indonesia; 2010. p. 508.
19. Adonogianaki E, Moughal NA, Kinane DF. Lactoferrin in the gingival crevice as a marker of polymorphonuclear leucocytes in periodontal diseases. J Clin Periodontol 1993; 20(1): 26-31.
20. Kusnoto. Karakterisasi molekular protein toxocara cati dan toxocara canis untuk pengembangan diagnostik toxocariasis. Disertasi. Surabaya: Program Pascasarjana Universitas Airlangga; 2008.