

Research Report

***Prevotella intermedia* and *Porphyromonas gingivalis* in dental caries with periapical granuloma**Risya Cilmiaty,¹ Afiono Agung Prasetyo,^{2,3,4} Khilyat Ulin Nur Zaini,^{3,4} Mandojo Rukmo,⁵ Suhartono Taat Putra⁶ and Widya Asmara⁷¹Department of Dental and Oral Disease, Faculty of Medicine, Universitas Sebelas Maret, Surakarta-Indonesia²Department of Microbiology, Faculty of Medicine Universitas Sebelas Maret, Surakarta- Indonesia³A-IGIC Research Group, Universitas Sebelas Maret, Surakarta-Indonesia⁴Center of Biotechnology and Biodiversity Research and Development, Universitas Sebelas Maret, Surakarta-Indonesia⁵Department of Conservative Dentistry, Faculty of Dentistry Universitas Airlangga, Surabaya-Indonesia⁶Department of Pathobiology, Faculty of Medicine Universitas Airlangga, Surabaya-Indonesia⁷Department of Microbiology, Faculty of Veterinary Medicine Universitas Gadjah Mada, Yogyakarta-Indonesia**ABSTRACT**

Background: Dental caries with necrotic pulp is a multifactorial disease that attacks enamel involving tooth pulp. The anaerobic bacteria infection in the pulp chamber could induce the formation of periapical granuloma. However, the presence of the most frequently anaerobic bacteria identified in apical periodontitis, *Porphyromonas gingivalis* and *Prevotella intermedia*, in periapical granuloma have not been confirmed. **Purpose:** The aims of study were to determine the presence of *Porphyromonas gingivalis* and *Prevotella intermedia* in dental caries with necrotic pulp and to determine its relation to periapical granuloma. **Methods:** Thirty-six patients of dental caries with necrotic pulp in Dr. Moewardi General Hospital in Surakarta, Indonesia were involved and classified into two groups, the group of patients with periapical granuloma and the group of patients without periapical granuloma. The caries tooth was extracted, and the chronic periapical tissue was swabbed and cultured on blood agar medium in anaerobic condition. The bacterial DNA was extracted from the positive cultures and subjected for Polymerase Chain Reaction (PCR). **Results:** Periapical granuloma was more likely found in women (OR 5.5, 95% CI=1.277-23.693; RR 2.5, 95% CI= 1.025-6.100). Black colonies bacteria were associated with periapical granuloma (OR 2.2, 95% CI=0.517-9.594; RR 1.5, 95% CI=0.653-3.623). *Porphyromonas gingivalis* and *Prevotella intermedia* were detected in group with or without periapical granuloma, however, only *Prevotella intermedia* was associated with periapical granuloma (OR 1.6, 95% CI=0.418-5.903; RR 1.3, 95% CI=0.653-2.393). **Conclusion:** The presence of *Porphyromonas gingivalis* and *Prevotella intermedia* in periapical granuloma were confirmed, however, only *Prevotella intermedia* were associated with periapical granuloma.

Key words: Dental caries, necrotic pulp, periapical granuloma, *Prevotella intermedia*, *Porphyromonas gingivalis*

ABSTRAK

Latar belakang: Karies gigi dengan pulpa nekrosis adalah penyakit multifaktorial yang menyerang enamel hingga ruang pulpa gigi. Infeksi bakteri anaerob di pulpa nekrosis dapat menginduksi pembentukan granuloma periapikal. Namun, keberadaan bakteri anaerob yang paling banyak ditemukan di periodontitis apikal, *Porphyromonas gingivalis* dan *Prevotella intermedia*, di granuloma periapikal masih perlu diteliti. **Tujuan:** Penelitian ini bertujuan meneliti keberadaan bakteri *Porphyromonas gingivalis* dan *Prevotella intermedia*, di karies gigi dengan pulpa nekrosis dan menganalisis kaitannya dengan granuloma periapikal. **Metode:** Tiga puluh enam pasien karies gigi dengan pulpa nekrosis di Rumah Sakit Umum Dr. Moewardi di Surakarta Indonesia dilibatkan dan diklasifikasikan dalam dua kelompok yaitu kelompok pasien dengan granuloma periapikal dan kelompok pasien tanpa granuloma periapikal. Gigi karies diekstraksi dan jaringan periapikal kronis diusap dan dikultur di media agar darah dalam kondisi anaerob. DNA bakteri diekstrak dari kultur yang positif dan dilakukan pemeriksaan dengan Polymerase Chain Reaction (PCR). **Hasil:** Granuloma periapikal lebih banyak ditemukan pada wanita (OR 5,5, 95% CI= 1,277-23,693; RR 2,5, 95% CI= 1,025-6,100). Koloni bakteri berwarna hitam diasosiasikan dengan granuloma periapikal (OR 2,2, 95% CI= 0,517-9,594; RR 1,5, 95% CI= 0,653-3,623). *Porphyromonas gingivalis* dan *Prevotella*

intermedia terdeteksi di jaringan karies dental dengan atau tanpa granuloma periapikal, namun hanya *Prevotella intermedia* yang diasosiasikan dengan granuloma periapikal (OR 1,6, 95% CI= 0,418-5,903; RR 1,3, 95% CI= 0,653-2,393). **Simpulan:** *Porphyromonas gingivalis* dan *Prevotella intermedia* ditemukan di granuloma periapikal, namun hanya *Prevotella intermedia* yang terkait langsung dengan granuloma periapikal.

Kata kunci: Karies gigi, pulpa nekrosis, granuloma periapikal, *Prevotella intermedia*, *Porphyromonas gingivalis*

Correspondence: Afiono Agung Prasetyo, c/o: Laboratorium Mikrobiologi Fakultas Kedokteran Universitas Sebelas Maret. Jl. Ir. Sutami 36 A Surakarta 57126, Indonesia. Email: afie.agp.la@gmail.com atau afieagp@yahoo.com

INTRODUCTION

Dental caries is characterized by the progressive demineralization of enamel, following acid metabolism by the bacteria.¹ The Indonesian population in range of age 15 years old or older, 71.2% had caries and 52.3% cases are untreated.² The untreated dental caries could lead into necrotic pulp, and infection of the root canal system resulting a disruption at the apical.³⁻⁵ In some case, the tooth become sensitive that is caused by hyperemia, edema and inflammation of the apical periodontal.⁶

Endodontic and periodontal infections are commonly found preceded by caries process and associated with anaerobic bacteria, including that of the black-pigmented bacteria, *Porphyromonas spp.* and *Prevotella spp.*⁷⁻⁹ The most frequently identified in acute and chronic apical periodontitis are *Porphyromonas gingivalis* and *Prevotella intermedia*.^{9,10} As gram-negative bacteria, both bacteria have lipopolysaccharide (LPS) in the cell wall, which can induce macrophage to release pro-inflammatory cytokines such as interleukin-8 (IL-8), IL-1 β , interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α), causing tissue inflammation and bone resorption.^{7,11} The bacteria also have exopolysaccharides (EPS) which can enhance their virulence.¹² *Porphyromonas gingivalis* (*P. gingivalis*) has occurred between family members¹³ and the bacteria presentation in periodontitis tissue also indicative of alveolar bone loss.¹⁴ *Prevotella intermedia* (*P. intermedia*) has ability to invade and evade the host innate response, so the possibility of infection will be increased.¹⁴ However,

the role of both bacteria in periapical granuloma have not been elucidated.

The presence of periodontal pathogen may affected by ethnic and geography.¹³ In Indonesia, the presence of *P. gingivalis* and *P. intermedia* are quite high in oral cavity, but there is lack information concerning the bacteria of chronic periapical tissue due to dental caries. The aims of study were to determine the presence of *Porphyromonas gingivalis* and *Prevotella intermedia* in dental caries with necrotic pulp and to determine its relation to periapical granuloma.

MATERIALS AND METHODS

Chronic periapical tissue of permanent teeth from dental caries patients aged 17-57 years old with normal albumin levels and no anemia at Dr. Moewardi General Hospital, in Surakarta, Indonesia were assayed in the study. All patients had no antibiotic and or immunosuppressant therapy prior the tooth extraction. None patient had systemic disease. The sample size was 9 in every group, calculated as previously published.¹⁶ The sample used was doubled for each group. Finally, the total sample used was 36, were classified into two groups, with and without periapical granuloma, based on following dental radiographic criteria: well-circumscribed radiolucent periapical lesion attached to the root apex and measured less than 1 cm^{6,17} (Figure 1A and 1B).

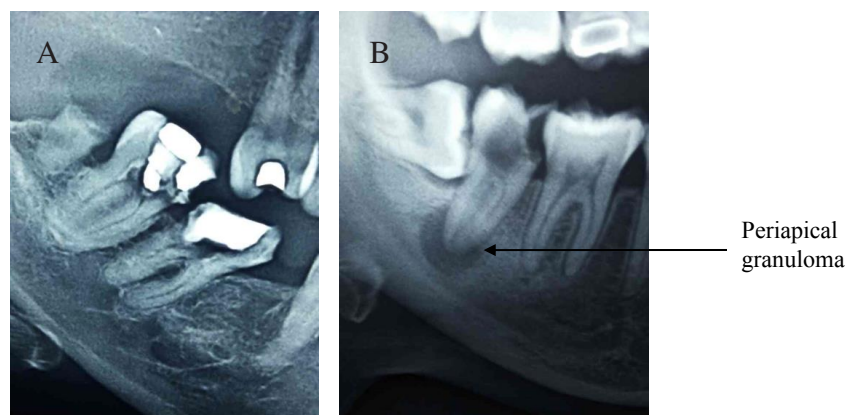
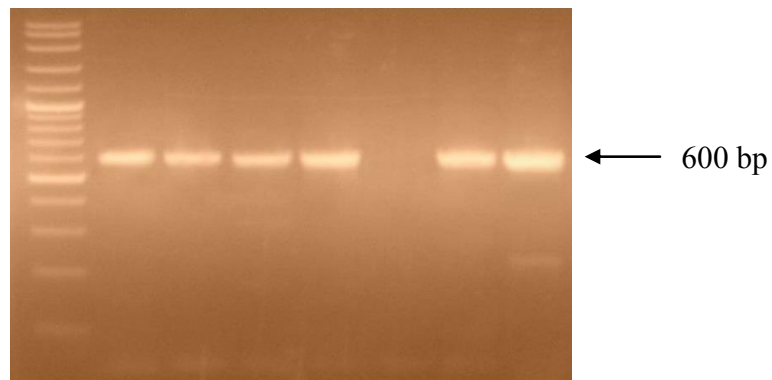
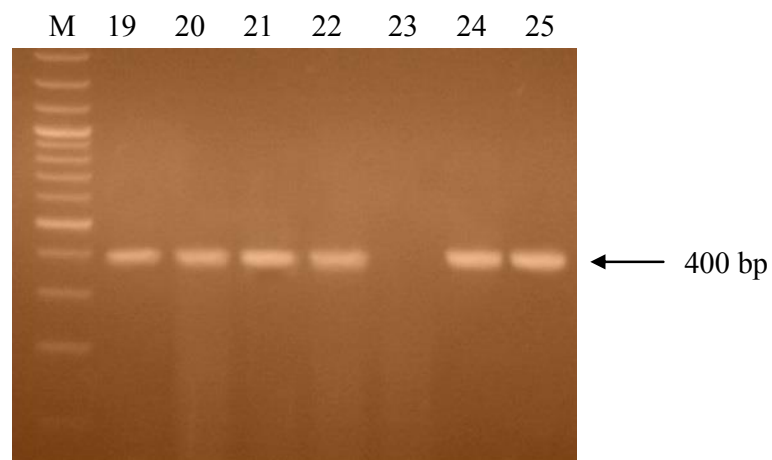


Figure 1. The radiographic of tooth (A) without periapical granuloma, (B) with periapical granuloma (radiolucent).

Table 1. Age, gender, dental element and radiographic of dental caries patients in General Hospital Dr. Moewardi in Surakarta.

		With periapical granuloma (n= 18)		Without periapical granuloma (n= 18)	
		N	%	N	%
Age	<21	5	27.8	4	22.2
	21-30	1	5.6	2	11.1
	31-40	8	44.4	2	11.1
	41-50	2	11.1	8	44.4
	>50	2	11.1	2	11.1
Gender	Men	4	22.2	11	61.1
	Women	14	77.8	7	38.9
Element	Quadrant 1	0	-	1	5.6
	Quadrant 2	4	22.2	7	38.9
	Quadrant 3	7	38.9	3	16.7
	Quadrant 4	7	38.9	7	38.9

**Figure 2.** A sample of electrophoresis result from PCR assay to detect the presentation of *P. gingivalis*'s DNA. PCR results from sample no. 19-25 were shown. M= VC 100bp Plus DNA Ladder (Vivantis, Selangor, Malaysia). *P. gingivalis*'s DNA was detected in sample no. 19-22 and 24-25.**Figure 3.** A sample of electrophoresis result from PCR assay to detect the presentation of *P. intermedia*'s DNA. PCR results from sample no. 19-25 were shown. M= VC 100bp Plus DNA Ladder (Vivantis). *P. intermedia*'s DNA was detected in sample no. 19-22 and 24-25.

Approval was obtained from institutional ethical committee review boards of the Faculty of Medicine of Universitas Sebelas Maret and Dr. Moewardi General Hospital in Surakarta, Indonesia. Informed consent was obtained from all patients involved in the study. Data including age, gender, dental elements and dental radiographic were obtained from all patients.

The caries tooth from each patient was extracted and the chronic periapical tissue was swabbed. The swab was then smeared on blood agar medium and incorporated into anaerobic jar with Gas generating kit (Thermo Scientific, Hampshire, United Kingdom) at 37° C for 7-14 days. The present study was focused on *P. gingivalis* and *P. intermedia*, so only the black-pigmented bacteria colonies' DNA were extracted using a High Pure PCR Template Preparation Kit (Roche Molecular Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The *P. gingivalis*' DNA was detected using 5' - AGG CTT CAG GCC ATA CTG CG - 3' and 5' - ACT GTT AGC AAC TAC CGA TGT - 3' set primer. Briefly, the PCR reaction was performed by initial denaturation at 94°C for 5 minutes, 40 cycles of 94° C for 45 seconds, 66°C for 1 minute and 72° C for 2 minutes, followed by a final extension at 72° C for 10 minutes. The *P. intermedia*'s DNA was detected using 5' - TTT GTT GGG GGG GAG TAA AGC - 3' and 5' - TCA ACA TCT CTG TAT CCT GCG T - 3' set primer. The PCR reaction was performed by initial denaturation at 94° C for 5 minutes, 40 cycles of 94° C for 45 seconds, 62°C for 1 minute and 72° C for 2 minutes followed by a final extension at 72° C for 10 minutes. All PCR reaction was performed using Ready To Go PCR Bead (Promega, Buckinghamshire, United Kingdom). The PCR products were then subjected to electrophoresis in 1% agarose gels, stained with Ethidium Bromide, and visualized under ultraviolet illumination. The data was analyzed by SPSS version 16 software (SPSS, Chicago, IL). A 95% confidence interval (CI) was used for all data analysis.

RESULTS

Thirty-six dental caries patients (19 women and 17 men) with necrotic pulp were agreed to participate. The mean age of the patients studied was 35.7 years old (in range of 17 to 57 years old) and the most frequent element was derived from 4th quadrant (38.9%, 14/36). Periapical granuloma was found in 77.8% (14/18) women (OR 5.5, 95% CI= 1.277-23.693; RR 2.5, 95% CI= 1.025-6.100) and 22.2% (4/18) men (OR 0.2; 95% CI= 0.042-0.783; RR 0.400; 95% CI= 0.164-0.976) (Table 1).

Twenty-five of the 36 (69.4%) samples had black-pigmented bacteria. These bacteria were identified in 77.8% (14/18) periapical granuloma's samples (OR 2.2, 95% CI= 0.517-9.594; RR 1.3, 95% CI=0.817-1.983) and 61.1% (11/18) of non periapical granuloma's samples (OR 0.4, 95% CI= 0.104-1.934; RR 0.8, 95% CI= 0.504-1.224). *P.*

gingivalis's DNA was detected (Figure 2) in 64.3% (9/14) of black colonies from periapical granuloma's samples and 63.6% (7/11) of black colonies from non periapical granuloma's samples ($p= 1.00$) and had no association with periapical granuloma formation (OR 1.0, 95% CI= 0.271-3.694). *P. intermedia*'s DNA was detected (Figure 3) in 57.1% (8/14) of black colonies from periapical granuloma's samples (OR 1.6, 95%CI= 0.418-5.903; RR 1.3, 95% CI=0.653-2.393). Co-infection of *P. gingivalis* and *P. intermedia* was detected only in 33.3% (6/18) periapical granuloma's samples (OR 1.0, 95% CI= 0.250-3.999; RR 1.0, 95% CI= 0.500-1.999).

DISCUSSION

Dental caries with necrotic pulp is a multi-causal disease that attacks the enamel and reach tooth pulp chamber, causing destruction structure of the tooth, and open the door to bacterial infection.^{1,4} The bacterial infection can activate host immune response to isolate and eradicate microorganisms or chronic irritants, causing periapical granuloma, an inflammatory reaction in the apex of non-vital tooth. The inflammatory tissue of periapical granuloma (containing macrophages, polymorphonuclear leukocytes and lymphocytes) is covered by epithelial cells and ultimately results in destruction of the alveolar bone surrounding the tooth.^{7,8}

The *P. gingivalis* and *P. intermedia* already known dominant in primary endodontic infection due to its content, LPS.⁸ LPS induces pulp fibroblast and osteoblast to produce IL-8. The IL-8 then attracts and activates polymorphonuclear leukocytes to surround and kills the bacteria, stimulates osteoclast activity and causes pain symptom in periapical lesion. The LPS also activates macrophages through CD14 receptor to produce pro-inflammatory mediators, IL-1 and TNF- α . IL-1 then induces the production of IL-12, TNF- α , IFN- γ and IL-1 itself. In human periapical lesion, IL-1 β , one variant of IL-1, is predominant and stimulates T-lymphocytes; enhances bone resorption and inhibits bone formation. The macrophages and T-lymphocytes in the inflammatory tissue also produce TNF- α . The TNF- α activates the macrophages, T-lymphocytes and natural killer cells and also stimulates bone resorption. However, if the bacteria (*P. gingivalis* and or *P. intermedia*) are difficult to be eliminated, T-lymphocyte would lead B-lymphocyte activation, to synthesize the antibody to bacterial antigen. The bacterial-antibody formation will attract activated-macrophages to surround and phagocyte the formation. Finally, all reaction will causes periapical granuloma formation in the root of the tooth.^{7,18-20}

P. gingivalis and *P. intermedia* are frequently detected in intraoral⁷⁻⁹ and the numbers of these bacteria are increase in the disease site compared with healthy site.⁹ In the present study, *P. gingivalis* and *P. intermedia* could be isolated from dental caries tissue, both of with and without periapical granuloma, consistent with previous reports,^{7,8,21}

however, only *P. intermedia* was associated with periapical granuloma.

In the present study, periapical granuloma was more likely found in women than in men. LPS of bacteria induces production of IL-1 β and TNF- α .¹¹ These pro-inflammatory cytokines could be influenced by sex hormone. Sex hormone, especially estrogen, already known significantly reduces *P. gingivalis* bacteria compared with *P. intermedia*.²¹ Testosterone also could reduce the expression of TNF- α and IL-1 β , so reduce the inflammation process.^{9,21-23} The study revealed that the presence of *P. gingivalis* and *P. intermedia* were confirmed in periapical granuloma and could be isolated in dental caries tissue; however, only *P. intermedia* had association with periapical granuloma. Further studies are needed to confirm and extend our findings.

ACKNOWLEDGEMENT

This work was supported partially by grants from the APBN/DIPA UNS (No. 159a/UN27.11/PN/2013) and BOPTN UNS (No. 165/UN27.11/PN/2013).

REFERENCES

- Deljo E, Cavaljuga S, Mescovic B. Prevalence of dental caries in the municipality gorazde during the period 2007-2012. *Mater Sociomed* 2013; 25(3): 163-6.
- The Indonesian household health survey. Health profile of Indonesian 2004. Jakarta: Ministry of health republic of Indonesia; 2006. p. 19.
- Bjørndal L. The caries process and its effect on the pulp: The science is changing and so is our understanding. *Pediatr Dent* 2008; 30(3): 192-6.
- Martin F, Nadkarni M, Jacques N, Hunter N. Quantitative microbiological study of human carious dentine by culture and real-time PCR: Association of anaerobes with histopathological changes in chronic pulpitis. *J Clin Microbiol* 2002; 40(5): 1698-704.
- Gomes GB, Sarkis-Onofre R, Bonow ML, Etges A, Jacinto RC. An investigation of the presence of specific anaerobic species in necrotic primary teeth. *Braz Oral Res* 2013; 27(2): 149-55.
- Rajendran R. Shafer's textbook of oral pathology 6th ed. India: Elsevier; 2009. p. 482.
- Garcia CC, Sempere FV, Diago MP, Bowen EM. The post-endodontic periapical lesion: Histologic and etiopathogenic aspects. *Med Oral Patol Oral Cir Bucal* 2007; 12(8): 585-90.
- Narayanan L, Vaishnavi C. Endodontic microbiology. *J Conserv Dent* 2010; 13(4): 233-9.
- Estrela CR, Pimenta FC, Alencar AH, Ruiz LF, Estrela C. Detection of selected bacterial species in intraoral sites of patients with chronic periodontitis using multiplex polymerase chain reaction. *J Appl Oral Sci* 2010; 18(4): 426-31.
- Ge X, Rodriguez R, Trinh M, Gunsolley J, Xu P. Oral microbiome of deep and shallow dental pockets in chronic periodontitis. *PLoS One* 2013; 8(6): e65520.
- Corcoran MP, Meydeni M, Lichtenstein AH, Schaefer EJ, Dillar A, Lamou-Fava S. Sex hormone modulation of proinflammatory cytokine and C-reactive protein expression in macrophages from older men and postmenopausal women. *J Endocrinol* 2010; 206(2): 217-24.
- Yamanaka T, Yamane K, Furukawa T, Matshumoto-Mashimo C, Sugimori C, Nambu T, Obata N, Walker CB, Leung K, Fukushima H. Comparison of the virulence of exopolysaccharide-producing *Prevotella intermedia* to exopolysaccharide non-producing periodontopathic organism. *BMC Infect Dis* 2011; 11: 228.
- Van Winkelhoff AJ, Rijnsburger MC, Abbas F, Timmerman MF, Van der Weijden GA, Winkle EG, Van der Velden U. Java project on periodontal disease: a study on transmission of *Porphyromonas gingivalis* in a remote Indonesian population. *J Clin Periodontol* 2007; 34(6): 480-4.
- Chaves ES, Jeffcoat MK, Ryerson CC, Snyder B. Persistent bacterial colonization of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in periodontitis and its association with alveolar bone loss after 6 months of therapy. 2000; 27(12): 897-903.
- Kononen E, Paju S, Pussinen PJ, Hyvonen M, Tella PD, Suominen-taipe L, Knuuttila M. Population-based study of salivary carriage of periodontal pathogens in adults. *J Clin Microbiol* 2007; 45(8): 2446-51.
- Sastroasmoro S, Ismael S. Dasar-dasar metodologi penelitian klinis. Edisi 2. Jakarta: Sagung Seto; 2002. p. 258-64.
- Langland OE, Anglairs RP, Preece JW. Principle of dental imaging. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 2002. p. 413.
- Nair PNR. Pathogenesis of apical periodontitis and causes of endodontic failures. *Crit Rev Oral Biol Med* 2004; 15(6): 348-81.
- Yang LC, Huang FM, Lin CS, Liu CM, Lai CC, Chang YC. Induction of interleukin-8 gene expression by black-pigmented bacteroides in human pulp fibroblast and osteoblast. *Int Endod J* 2003; 36(11): 774-9.
- Graunaite I, Lodiene G, Maciulskiene V. Pathogenesis of apical periodontitis: a literature review. *J Oral Maxillofac Res* 2011; 3(4): e1.
- Tarkkila L, Kari K, Furuholm J, Tiitinen A, Meurman J. Periodontal disease-associated micro-organisms in peri-menopausal and post-menopausal women using or not using hormone replacement therapy. A two-year follow-up study. *BMC Oral Health* 2010; 10: 10.
- Blasco-Baque V, Serino M, Vergnes J, Riant E, Loubieres P, Arnal J, Gourdy P, Sixou M, Burcelin R, Kemoun P. High-fat diet induces periodontitis in mice through lipopolysaccharides (LPS) receptor signaling: Protective action of estrogens. *PLoS One*. 2012; 7(11): e48220.
- Saitoa A, Inagaki S, Ishihara K. Differential ability of periodontopathic bacteria to modulate invasion. *Microb Pathog* 2009; 47(6): 329-33.