BACTERIAL PATHOGENICITY

Expression of trypsin-like activity by the genera *Corynebacterium* and *Actinomyces* in canine periodontitis

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Trypsin-like activity (TLA), clinical parameters and TLA-positive bacteria were examined in periodontitis and healthy sites in dogs. TLA was markedly higher in periodontitis than at healthy sites. There was good correlation between TLA positivity and severity of periodontal disease. The proportions of TLA-positive bacteria to total isolates in periodontitis and healthy sites were 21.1% and 2.1%, respectively. Among TLA-positive bacteria in periodontitis sites, 4.4% showed strong TLA activity, 35.3% showed moderate and 60.3% showed weak activity. In the healthy sites, all the TLA-positive bacteria showed weak activity. In all, 90% of the total number of TLA-positive bacteria were identified as belonging to the family Actinomycetaceae; 40% of bacteria belonging to the family Actinomycetaceae were identified as genus *Corynebacterium* with moderate trypsin-like activity and the remaining 60% were identified as genus *Actinomyces* with weak activity. Obligately anaerobic bacteria accounted for only 5.9% of the total population of TLA-positive bacteria; they were gram-negative coccobacilli, gram-positive rods and gram-positive cocci. These observations suggested that bacteria in the family Actinomycetaceae may play an important role in periodontitis and that measurement of TLA is a clinically reliable marker for the diagnosis of periodontitis in dogs.

Introduction

Periodontitis is an infectious disease caused primarily by micro-organisms that affect the supporting tissues of the teeth. Black-pigmented, anaerobic, gram-negative rods such as *Porphyromonas* spp. and *Prevotella* spp. have been implicated as key pathogenic and causative agents of periodontitis in both man and dogs [1–5]. Bacteria produce various kinds of enzymes, metabolic end-products and toxins that directly damage periodontal tissues, resulting in bone loss. Some of the periodontal bacteria such as *Por. gingivalis*, *Bacteroides forsythus*, *Treponema denticola* and some *Capnocytophaga* spp. possess a trypsin-like enzyme and are considered to be periodontopathic [6–10]. These bacteria hydrolyse the synthetic trypsin substrates N-benzoyl-DL-arginine-2-naphthylamide (BANA) and N-carbobenzoxymethyl-glycyl-glycyl-arginyl-3,5-dibromo-4-hydroxyaniline (DBHA). BANA and DBHA have been used to develop rapid and simple methods for diagnosis

and monitoring of periodontal therapy in human periodontitis [6, 7].

The aim of the present study was to determine whether there is a relationship between the presence of trypsin-like activity (TLA)-positive bacteria and periodontitis and to isolate and characterise TLA-positive bacteria in dogs.

Materials and methods

Subjects and clinical examination

Four female beagles, aged 3–7 years with periodontitis ranging from moderate to severe, and three periodontally healthy female beagles, aged 3–4 years, were studied. The dogs were housed in separate cages and fed a canine diet, with water available *ad libitum*. Dogs were generally healthy and had not received antibiotics before the study. Studies were performed in accordance with the ethical guidelines for animal experiments at the Animal Research Center of Nihon University School of Dentistry at Matsudo.

Eight periodontal pockets and six normal gingival

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crevices (two sites in each dog) were examined. The clinical parameters measured were: gingival crevicular fluid (GCF) – the volume of fluid in the pockets was determined by placing paper that absorbs GCF in the pocket for 5 s, then the moistened area was measured immediately with a Periotron (Harco Medical, Tustin, CA, USA); pocket depth (PD) – measured to the nearest mm with a standard periodontal probe; probing depth – measured at the deepest site of the periodontal pocket on the buccal surface of the tooth; and bleeding of the gums on probing (BOP, 0.5-mm diameter probe), classified as negative or positive.

Measurement of TLA

TLA was measured by the SK-013 method [7], now commercially developed as the Periocheck periodontal diagnostic kit (Sunstar, Takatsuki, Japan). Briefly, three paper points were placed in the pocket for 30 s, then placed into a small vial containing substrate (DBHA), chromogen and ascorbic oxidase solution, followed by agitation for 10 s. The mixture was then incubated at 37°C for 15 min. The OD was measured spectrophotometrically at 666 nm. The enzymic activity was calculated from a standard curve and recorded as trypsin units (DBHA TU/ml).

The quantitative assay of TLA for cultivable bacteria was performed with BANA and N-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) as substrates as described previously [8, 11]. Briefly, 100 µl of BANA (1.0 mM) or BAPNA (1.8 mM) were added to 100 µl of the various bacterial cell suspensions in the wells of a plastic microtitration plate (Iwaki Scitex DIV, Chiba, Japan). The mixtures were incubated at 37°C for 4 h, and the colour was developed by additions of 10 µl each of SDS 10% w/v in 2 M Tris/HCl, pH 8.0, and Fast Blue BB salt 0.35% w/v in 2-methoxyethanol solution only for BANA. The absorbances at 492 nm for BANA and at 415 nm for BAPNA were measured with a microplate reader (Corona Electric, Ibaragi, Japan). TLA-negative bacteria showed absorbance of 0 at 492 nm or 415 nm when the cell suspension with an OD₅₄₀ value of 1.0 was used. TLA was calculated in trypsin units (BANA or BAPNA TU/ml), with trypsin (Wako Pure Chemicals, Japan) as a standard. The qualitative TLA was performed by the API-ZYM system (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions.

Microbiological examination

GCF was obtained by placing paper points in each site for 10 s, which were then quickly transferred to 500 µl of reduced transport fluid [12] and immediately placed in an anaerobic glove box (Forma Scientific anaerobic system model 1024). Each sample was sonicated, diluted and plated on Gifu Anaerobic Medium (Nissui, Tokyo, Japan) agar with rabbit blood 5% v/v and containing haemin 0.0005% w/v and menadione 0.0001% w/v for culture. The plates were incubated at 37°C for 5 days. The numbers of colonies were counted and total viable bacterial numbers were determined.

Approximately 40 well separated colonies were picked at random and subcultured on pairs of identical plates, and one of each pair was incubated anaerobically and the other aerobically. All isolates were characterised by Gram's stain and TLA assay. The identities of the isolates were determined with the commercial API-CORYNE, API-ANAEROBE, API ZYM and API 50CH systems (bioMérieux) following the manufacturer's instructions. For TLA assay, the cells were harvested from plates, washed three times in Sorensen buffer [8] and resuspended in the same buffer to give an OD₅₄₀ value of 1.0. Serial two-fold dilutions of the cell suspensions were prepared to determine the detection limits of the TLA assay. The bacterial cell numbers were also counted in an EKDS Chamber (Sugagaki Med, Tokyo, Japan).

Statistical analysis

The data were analysed statistically by calculating means and SDs. Differences between means of the periodontal and healthy sites were evaluated by Student's *t* test (Excel version 5.0; Microsoft, WA, USA).

Results

The clinical parameters of the dogs with periodontitis and periodontally healthy sites are summarised in Table 1. Differences between periodontitis and healthy sites were significant for all clinical parameters.

Fig. 1 shows the relationships between the TLA and

Table 1. Clinical parameters of sampling sites in healthy dogs and dogs with periodontitis

Group	Number of dogs	Number of sampling sites	Pocket depth* (mm)	GCF value*	Frequency of bleeding on probing
Healthy	3	6	2.16 (1.04) [†]	38.0 (9.6) [†]	0/6 [†]
Periodontitis	4	8	5.25 (1.47) [†]	116.8 (25.3) [†]	7/8 [†]

*Mean (SD).

[†]*p* < 0.005.

Fig. 1. Relationship between frequency of TLA-positive bacteria in total isolates and TLA seen with Periocheck kit (DBHA as a substrate). □, percentage of TLA-positive bacteria; ▨, TLA. Bars, SD.

frequency of TLA-positive bacteria at periodontitis and healthy sites. The average recoveries of total cultivable bacteria were 1×10^8 cfu/ml at periodontitis sites and 1×10^7 cfu/ml at healthy sites, respectively. TLA-positive bacteria were found to represent 21.1% of the total isolates at periodontitis sites. In contrast, healthy sites showed only 10% of this number of TLA-positive bacteria. TLA values measured by a Periocheck kit (ability to hydrolyse DBHA) were 1.16 and 0.21 DBHA TU/ml in periodontitis and healthy sites, respectively.

Fig. 2 shows the standard curves of TLA prepared with BANA (Fig. 2a) and BAPNA (Fig. 2b) as substrates. Quantitative linear curves were obtained with 0.02–0.20 BANA TU/ml and 0.01–0.08 BAPNA TU/ml by the microplate assay. Both substrates were used for all cultivable bacteria isolated.

TLA-positive bacteria from both periodontitis and

healthy sites were isolated and TLA was measured with BANA as the substrate (Table 2). Weak, moderate and strong TLA corresponded to API-ZYM values of 1–2, 3–4 and >5, respectively. Of a total of 238 isolates from healthy sites, five showed weak activity and no moderate or strongly positive isolates were found. However, among 315 isolates from periodontitis sites, 41 showed weak TLA, 24 showed moderate TLA and three showed strong TLA.

Further characteristics of TLA-positive bacteria are summarised in Table 3. Approximately 90% of all TLA-positive isolates were filamentous, gram-positive and facultatively anaerobic bacteria. They were identified as belonging to the family Actinomycetaceae. Among these Actinomycetaceae isolates in periodontitis sites, 40% belonged to the genus *Corynebacterium* and showed moderate TLA of 1.75 SD 0.23 BANA TU/ml, and 60% belonged to the genus *Actinomyces* and showed weak TLA of 0.65 SD 0.29

Fig. 2. Standard curve for TLA obtained with (a) BANA, (b) BAPNA, as a substrate. The data are plotted as the mean (○) and SD.

Table 2. Distribution of TLA-positive bacteria

Group	Total	Number of isolates with TLA (BANA TU/ml)		
		Weak (0.2 ~ 1.0)	Moderate (1.0 ~ 2.0)	Strong (>2.0)
Healthy	238	5	0	0
Periodontitis	315	41	24	3

Table 3. Characterisation of TLA-positive bacteria from canine periodontal sites

Family/genus	Shape	Gram's stain	Anaerobiosis requirements	Mean (SD) TLA (U/ml)		Percentage of TLA-positive bacteria
				BANA	BAPNA	
Actinomycetaceae	Filamentous	...	...	...	...	...
<i>Actinomyces</i>	Some branching	+	Facultative	0.65 (0.29)	0.13 (0.06)	54.4 (100)*
<i>Corynebacterium</i>	Whip-like	+	Facultative	1.75 (0.23)	0.18 (0.05)	35.3
ND	Rods	+	Obligate	1.01	0.31	2.9
ND	Coccobacillus	—	Facultative	9.91	2.28	2.9
ND	Coccobacillus	—	Obligate	9.59	2.60	1.5
	Coccus					
ND	Pair	—	Facultative	1.19	0.54	1.5
ND	Chain	+	Obligate	0.99	0.48	1.5

ND, not determined.

*Number in parentheses indicates isolates from healthy site.

BANA TU/ml. At the healthy sites, all TLA-positive bacteria were from the genus *Actinomyces* and had weak activity. These bacteria belonging to the family Actinomycetaceae also showed weak ability to hydrolyse BAPNA (<0.2 BAPNA TU/ml). Obligately anaerobic bacteria accounted for 5.9% of all TLA-positive bacteria, and one showed strong TLA – 9.59 BANA TU/ml and 2.60 BAPNA TU/ml. The others had weak TLA. Obligately anaerobic bacteria were found only in periodontitis sites. The bacteria with highest hydrolytic activity for BANA were a gram-negative, facultatively anaerobic coccobacillus with TLA values of 9.91 BANA TU/ml and 2.28 BAPNA TU/ml; there was only one isolate and it was from a periodontitis site. A gram-negative, facultative anaerobic diplococcus with weak TLA was also found in periodontitis sites.

Discussion

Periodontal disease is the most common oral disease in dogs [13, 14]. There have been many investigations concerning periodontitis in dogs and man [1, 8, 14]. The accumulation of plaque and calculus on tooth surfaces, particularly at the gingival margin in dogs, causes an increase in the number of micro-organisms within the gingival pocket and progression and development to periodontitis. Diagnostic tests for human periodontal infections have been developed based on the hydrolysis of trypsin-like enzyme substrates BANA and DBHA by micro-organisms in subgingival plaque and crevicular fluid [7, 15, 16]. However, there are very few BANA-positive species among the cultivable plaque flora [17]. The three species, *Por. gingivalis*, *B. forsythus* and *T. denticola*,

are always positive for BANA hydrolysis [6, 7, 9]. Other BANA hydrolytic species in man were reported to include some *Capnocytophaga* spp. [8] and Actinomycetaceae [18].

The present investigation applied a diagnostic test to periodontal disease in dogs. This study extended earlier findings by comparison of the TLA test for dogs with a commercially available Periocheck kit. The enzymic activity was higher in disease sites than in healthy sites (Fig. 1). The recoveries of total cultivable and TLA-positive bacteria at periodontal sites were both 10-fold higher than those at healthy sites, i.e., the numbers of TLA-positive bacteria at periodontal sites were 100-fold greater than those at healthy site. The magnitude of DBHA-hydrolysing activity was correlated with proportions of TLA-positive bacteria, and with the periodontal status of the subjects (Tables 1 and 2). Isolation and characterisation of TLA-positive bacteria yielded several different bacteria (Table 3). Although *Por. gingivalis* from man has strong TLA, *Porphyromonas* spp. isolated from dogs do not produce trypsin-like enzymes [2, 4, 19]. Some studies [3, 20] have reported that black-pigmented, anaerobic gram-negative rods from periodontitis in dogs have TLA. However, genotypic characterisation is needed to identify the genus and species of these isolates. Furthermore, there have been few reports concerning *B. forsythus*, spirochaetes, *Capnocytophaga* spp. and Actinomycetaceae from dogs. Human isolates of these bacteria have trypsin-like enzymes. Some of the weakly TLA-positive bacteria found in the present study, which were gram-negative coccobacilli (Table 3), might have been similar to the organisms described by Karjalainen *et al.* [3]. However, these strains showed no pigmentation. In

the present study, 90% of the TLA-positive bacteria were members of the Actinomycetaceae (Table 3). These results indicate that members of the family Actinomycetaceae are major contributors to TLA in the gingival pocket in dogs.

The progression and development of periodontitis in dogs might be different from those in man. Plaque and calculus formation in dogs became more severe than that in man. *C. matruchotii*, which has the strongest calculus-forming ability, is very important not only in supragingival plaque but also in subgingival plaque and crevicular fluid [21, 22]. Colonies of whip-like bacteria with moderate TLA (40% of all Actinomycetaceae) were rough, tough and adherent to the medium, and were identified as belonging to the genus *Corynebacterium*. They were similar in shape to *C. matruchotii* (data not shown). The other 60% of the Actinomycetaceae bacteria were identified as belonging to the genus *Actinomyces*. The determination of the species of these bacteria must await genetic analysis. It is of interest that Actinomycetaceae from man, *R. dentocariosa* and *C. matruchotii*, produce trypsin-like enzyme [17] and *C. matruchotii* is stimulated to grow under anaerobic conditions by haemin [22]. In dogs, filamentous bacteria such as Actinomycetaceae might play the same role as *Por. gingivalis* in human periodontitis.

The use of chromogenic substrates for rapid detection of bacterial enzymic activities is a useful method for diagnosing and monitoring different periodontal conditions in dogs. The present findings can be summarised as follows: BANA hydrolysis was directly related to the number of periodontopathic bacteria in crevicular fluid; TLA positivity is a risk indicator for dogs associated with colonisation by the TLA-positive bacteria, increased probing depth and active periodontitis; and bacteria of the genera *Corynebacterium* and *Actinomyces* contribute to TLA.

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