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Highly Specific Protease-Based Approach for Detection of *Porphyromonas gingivalis* in Diagnosis of Periodontitis

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***Porphyromonas gingivalis* is associated with the development of periodontitis. Here we describe the development of a highly specific protease-based diagnostic method for the detection of *P. gingivalis* in gingival crevicular fluid. Screening of a proteolytic peptide substrate library, including fluorogenic dipeptides that contain D-amino acids, led to the discovery of five *P. gingivalis*-specific substrates. Due to the presence of lysine and arginine residues in these substrates, it was hypothesized that the cleavage was mediated by the gingipains, a group of *P. gingivalis*-specific proteases. This hypothesis was confirmed by the observation that *P. gingivalis* gingipain knockout strains demonstrated clearly impaired substrate cleavage efficacy. Further, proteolytic activity on the substrates was increased by the addition of the gingipain stimulators dithiothreitol and L-cysteine and decreased by the inhibitors leupeptin and *N*-ethylmaleimide. Screening of saliva and gingival crevicular fluid of periodontitis patients and healthy controls showed the potential of the substrates to diagnose the presence of *P. gingivalis* proteases. By using paper points, a sensitivity of approximately 10^5 CFU/ml was achieved. *P. gingivalis*-reactive substrates fully composed of L-amino acids and Bz-L-Arg-$NHPhNO_2$ showed a relatively low specificity (44 to 85%). However, the five *P. gingivalis*-specific substrates that each contained a single D-amino acid showed high specificity (96 to 100%). This observation underlines the importance of the presence of D-amino acids in substrates used for the detection of bacterial proteases. We envisage that these substrates may improve the specificity of the current enzyme-based diagnosis of periodontitis associated with *P. gingivalis*.**

Periodontitis is an inflammation of the periodontium, the tissues that surround and support the teeth. Severe periodontitis affects at least 10% of the general population and involves progressive loss of the alveolar bone around the teeth (17, 18, 29). If left untreated, periodontitis can lead to loosening and subsequent loss of teeth (45). Periodontitis is induced by microorganisms that adhere to and grow at the gingival margin, along with an overly aggressive immune response against these microorganisms (9). The microorganisms implicated in periodontitis include *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Tannerella forsythia*, and *Treponema denticola* (11, 13, 26, 31). Periodontal pathogens, in particular, *T. denticola*, *T. forsythia*, and *P. gingivalis*, secrete protease virulence factors that allow these bacteria to invade the host's tissues (13, 16, 21, 25, 37). By liberating amino acids from host proteins, secreted proteases are actively involved in the (anaerobic) metabolism of these bacteria. In addition to being a major cause of tooth loss, periodontal disease has also been associated with several systemic diseases. Animal- and population-based studies have demonstrated associations between periodontal disease and diabetes, cardiovascular disease, rheumatoid arthritis, stroke, and adverse pregnancy outcomes (4, 8, 38). Although the mechanisms that cause these associations are far from being completely understood, more reliable detection of early stage periodontal disease could have widespread health benefits, even beyond the prevention of tooth loss.

Despite today's widespread occurrence of periodontal disease, currently available diagnostic tests are limited in their sensitivity and specificity (46). The best available diagnostic aid is measurement of the depth of the tooth pocket, but this provides a retrospective analysis only and mostly when tooth attachment is already lost. Moreover, this is a mere effect measurement and this does not help to identify causative agents. Culture-based, nucleic acid-based, and antibody-based diagnostic methods are available to help identify the periodontal pathogens involved, which is important to determine suitable therapeutic procedures invoking the highest chance of therapeutic success (6, 14, 19). Still, cultivation or using nucleic acid-based and immunochemical tests to identify periodontal pathogens can be very laborious and time-consuming. So far, direct detection and identification of periodontal pathogens *in situ* have proven difficult.

Therefore, the goal of this study was to develop a rapid and simple diagnostic technology that would enable the dental practitioner to perform a "chair-side" test to identify the presence of periodontal pathogens with a focus on *P. gingivalis*. The application of such a test based on the enzymatic diagnosis of periodontal pathogens has already been described by Loesche and coworkers (27). Using the benzoyl-DL-arginine-naphthylamide (BANA) substrate, it is possible to detect *T. denticola*, *T. forsythia*, and *P. gingivalis* by their proteolytic activity in plaque samples (28). To start suitable treatment of periodontitis, it is important to know

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TABLE 1 Bacterial strains used in this study

Strain	Characteristic	Reference(s)
Porphyromonas gingivalis W50		
Porphyromonas gingivalis HG91	Nonencapsulated K$^-$	23, 24
Porphyromonas gingivalis W83	Capsular serotype K1	23, 24
Porphyromonas gingivalis HG184	Capsular serotype K2	23, 24
Porphyromonas gingivalis A7A1-28	Capsular serotype K3	23, 24
Porphyromonas gingivalis ATCC 49417	Capsular serotype K4	23, 24
Porphyromonas gingivalis HG1690	Capsular serotype K5	23, 24
Porphyromonas gingivalis HG1691	Capsular serotype K6	23, 24
Porphyromonas gingivalis 34-4	Capsular serotype K7	3
Porphyromonas gingivalis K1A	ΔKgp	1
Porphyromonas gingivalis KDP133	ΔRgpA ΔRgpB	41
Porphyromonas gingivalis KDP136	ΔKgp ΔRgpA ΔRgpB	41
Actinomyces naeslundii ATCC 12104		
Actinomyces odontolyticus HG472		
Aggregatibacter actinomycetemcomitans NCTC 9710		
Fusobacterium nucleatum subsp. *periodontium* ATCC 33693		
Fusobacterium nucleatum subsp. *nucleatum* ATCC 25586		
Fusobacterium nucleatum subsp. *polymorphum* ATCC 10953		
Peptostreptococcus micros ATCC 33270		
Prevotella intermedia ATCC 25611		
Prevotella nigrescens NCTC 9336		
Streptococcus mitis I SK 95		
Streptococcus oralis SK 1477		
Tannerella forsythia ATCC 43037		
Treponema denticola ATCC 35405		

which pathogen is involved. Nevertheless using the BANA substrate, it is not possible to distinguish among these three bacterial species and exclusively detect *P. gingivalis*.

We showed in a previous study that the introduction of a D-amino acid residue in a peptide substrate enhanced the specificity of a test that was geared toward the detection of proteolytic activity specifically associated with a certain bacterial species (20). The all-L-amino-acid peptide fluorescein isothiocyanate (FITC)-Leu-Leu-Dabcyl (KDbc) was cleaved by a wide range of bacterial proteases. However, when one of the leucines within the substrate was substituted by its D-enantiomer, the peptide was exclusively cleaved by *Bacillus* ssp. and not by any of the other species tested (20). Further, in contrast to the all-L-amino-acid parental substrate, the variant was not degraded by enzymes present in serum and saliva. Essentially, the introduction of D-amino acids in a peptide aids to design substrates that are bacterial species specific due to their presence as a component of the bacterial cell wall (43). In contrast, D-amino acids are, with only a few exceptions, not metabolized in eukaryotic cells (33) In our view, this approach has opened a novel, attractive avenue to develop more bacterial species-specific substrates that can be applied in the diagnosis of infectious agents in complex matrices. Based on the study on D-amino-acid-containing peptides for the detection of *Bacillus* spp., we developed a substrate library containing fluorogenic dipeptides that contain no, one, or two D-amino acids. We used this approach to shotgun screen for substrates that react specifically with *P. gingivalis* proteases.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study are listed in Table 1. Bacteria were grown in 15 ml brain heart infusion (BHI) medium (Bio-Trading, Mijdrecht, The Netherlands) under anaerobic conditions at 37°C. All *P. gingivalis* cultures were supplemented with 1 μg/ml hemin and 0.5 μg/ml menadione (Sigma, Zwijndrecht, The Netherlands). After 72 h of culturing, the bacteria were pelleted by centrifugation for 10 min at 10,000 × *g*. The supernatant, containing secreted enzymes, was sterilized by filtration through a 0.22-μm filter (Millipore, Amsterdam, The Netherlands). Crude samples were used immediately or stored at −20°C for later use.

Fluorescence resonance energy transfer (FRET) substrates. The 115 novel fluorogenic substrates used were purchased at PepScan Presto B.V. (Lelystad, The Netherlands) and were >90% pure (20, 36). The identity of the substrates was confirmed by mass spectrometry. The *P. gingivalis*-specific substrates were designated "BikKams" (Table 2). Assays were performed in Blackwell clear-bottom 96-well plates (Corning, Lowell, MA). Proteolytic activity was determined by the addition of 1 μl of substrate (800 μM) to 50 μl of filtered culture supernatant or whole culture for the sensitivity assay. Culture broth was used as a negative control. Plates were read for 60 min at 37°C with 2-min intervals on a fluorescence microplate reader (Fluostar Galaxy, BMG Laboratories, Offenburg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Relative fluorescence (RF) values were obtained after correction against the culture broth control. The protease activity was defined in RF per minute (RF/min). An RF/min value higher than 5 was considered positive.

TABLE 2 Sequences of FRET library peptides cleaved by *P. gingivalis* culture supernatant

Peptide	Sequence
BikKam9	FITC–Arg–D-Asp–KDbc
BikKam10	FITC–Arg–D-Glu–KDbc
BikKam11	FITC–Arg–D-His–KDbc
BikKam12	FITC–Arg–D-Lys–KDbc
BikKam13	FITC–Arg–D-Arg–KDbc
BikKam14	FITC-Lys-Lys-KDbc
BikKam15	FITC-Phe-Arg-KDbc
BikKam16	FITC-Arg-Arg-KDbc

Cleavage characteristics of *P. gingivalis* substrates. *P. gingivalis* strain W50 culture supernatant was prepared as described above. Culture supernatant was incubated with 16 μM of each substrate in the presence of various concentrations of five different chemicals which are known to influence the cleavage activity of gingipains (5, 7). Proteolytic activity was measured as described previously. The compounds used were leupeptin, dithiothreitol (DTT), *N*-ethylmaleimide (NEM), L-cysteine and glycylglycine. All chemicals were obtained from Sigma (Zwijndrecht, The Netherlands).

Sensitivity testing of *P. gingivalis* substrates. *P. gingivalis* W50 was cultured in BHI medium supplemented with 1 μg/ml hemin and 0.5 μg/ml menadione for 72 h under anaerobic conditions at 37°C. The number of bacteria was determined by plating 10-fold serial dilutions on Trypticase soy agar plates (BioTrading, Mijdrecht, The Netherlands). Plates were incubated at 37°C under anaerobic conditions, and bacteria were enumerated after 3 days of incubation. The culture was serially diluted in culture broth (10^9, 10^8, 10^7, 10^6, and 10^5 CFU/ml), and 50 μl of each dilution was used to test the sensitivities of the substrates. Cleavage of the substrates was catalyzed by the addition of 5 μl of L-cysteine (50 mM) to each enzyme reaction mixture.

To mimic clinical samples, paper points were spiked with various concentrations of *P. gingivalis* W50. For this purpose, a *P. gingivalis* culture was serially diluted as described above. Paper points were incubated in the diluted culture until saturated and placed into reduced transport fluid (RTF)-containing vials (44). Four paper points per dilution were used. After thorough vortexing, 50 μl of suspension was incubated with 1 μl of substrate (800 μM) and 5 μl of L-cysteine (50 mM).

Proteolytic activity was measured as described previously. RF values were obtained after correction against the culture broth or RTF control. The protease activity was defined in RF/min. An RF/min value higher than 5 was considered positive.

Analysis of periodontitis patient paper points. To study the clinical applicability of the substrates to diagnose *P. gingivalis* infections *in situ*, gingival crevicular fluid from 72 patients suffering from periodontitis, sampled using paper points, was utilized. The study was approved by the Institutional Ethical Board of the Academic Hospital Vrije Universiteit at Amsterdam, and informed consent was obtained from all donors. Subgingival samples were taken from four different periodontal pockets per patient using paper points. After sampling, the paper points were transferred into RTF-containing vials. After thorough vortexing, 1 μl of substrate (800 μM) and 5 μl of L-cysteine (50 mM) were added to 50 μl of suspension. Plates were read for 90 min at 37°C with 2-min intervals on a fluorescence microplate reader as described previously. RF values were obtained after correction against the RTF control. The protease activity was defined in RF/min.

Additionally, the RTF containing dental plaque bacteria was analyzed using Bz-L-Arg-$NHPhNO_2$ (L-BApNA; PeptaNova, Sandhausen, Germany), a commercially available proteolytic substrate which is commonly used for the diagnosis of periodontal pathogens. For this purpose, 50 μl of suspension was incubated with 4 mM L-BApNA and 5 mM L-cysteine. Plates were incubated at 37°C for 60 min, and optical density at 405 nm (OD_{405}) was measured using a microplate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands). Paper points with an RF/min value of >5 or an OD_{405} of >1.2 were considered positive. Simultaneously, serving as the gold standard, gingival crevicular fluid eluted from the paper points from the same pockets was analyzed for the presence of a number of typical periodontal pathogens, including *A. actinomycetemcomitans*, *P. intermedia*, *T. forsythia*, *Peptostreptococcus micros*, *Fusobacterium nucleatum*, and *P. gingivalis*, using standard microbiological culture. Sensitivity is defined as the percentage of samples that are BikKam or L-BApNA positive as well as *P. gingivalis* culture positive. Specificity is defined as the percentage of samples which are both BikKam or L-BApNA negative and *P. gingivalis* culture negative (<10 CFU/ml).

RESULTS

Design of a FRET substrate library. We developed a shotgun substrate library based on our study on D-amino-acid-containing peptides for the detection of *Bacillus* spp. (20). The library consisted of fluorogenic substrate peptides, each comprising two amino acids where (i) both amino acids were L-amino acids, (ii) the C-terminally located amino acid was a D-amino acid, and (iii) both amino acids were D-amino acids. All peptides were flanked with an amino hexanoic acid-linked FITC probe at the N terminus and a lysine-coupled KDbc quencher at the C terminus. The library consists of 115 peptides in total (see Table S1 in the supplemental material).

To test the library concept, culture supernatant of *P. gingivalis* was incubated with all FRET peptides present within the library. Of the 115 peptides, 8 were cleaved by the *P. gingivalis* culture supernatant. Strikingly, all *P. gingivalis*-positive substrates contained an arginine or a lysine residue (Table 2).

Specificity of the *P. gingivalis* substrates. To examine the specificity of the *P. gingivalis*-positive substrates, the peptides were incubated with culture supernatants from eight different *P. gingivalis* strains and 12 other oral pathogens (Table 3). All eight substrates were cleaved by all of the *P. gingivalis* strains tested, except for BikKam9, which was not cleaved by the culture supernatant of *P. gingivalis* K2. Further, cleavage of the substrates varied in efficiency; BikKam9 and BikKam10 showed the lowest cleavage activity, whereas BikKam14 to BikKam16 showed the highest cleavage activity (Table 3). It was found that the majority of the substrates cleaved by *P. gingivalis* enzymes were not degraded by any of the other oral pathogens tested, the only exception being BikKam15, which was cleaved by proteases present in the culture supernatant of *T. forsythia* (Table 3).

To further evaluate the possibility of the use of these novel substrates in the diagnosis of periodontitis, saliva from seven healthy volunteers was incubated with the eight *P. gingivalis* substrates. As expected, the substrates which consisted of exclusively L-amino acids (BikKam14 to BikKam16) were cleaved by saliva whereas no cleavage activity was observed when the D-amino-acid-containing substrates were used (Table 3).

Mapping of the proteolytic characteristics of the *P. gingivalis* FRET substrates. Of the substrates present in the library, only the peptides which contained arginine or lysine residues were cleaved by the culture supernatant of *P. gingivalis*. From this observation, we hypothesized that the enzymes responsible for the cleavage of the substrates could be the *P. gingivalis*-specific Arg-gingipain and Lys-gingipain peptidases. These gingipains are members of the cysteine peptidase family. Therefore, we verified in addition whether (i) the addition of L-cysteine and DTT could stimulate cleavage and (ii) inhibitors such as NEM and leupeptin could inhibit proteolytic activity.

The addition of DTT and L-cysteine resulted in the increased degradation of all substrates (Table 4). Especially the presence of L-cysteine led to a significant increase in activity of BikKam14 and BikKam16 cleavage (>10- to 28-fold compared to the control). In line, decreased proteolytic activity was observed when the reaction was performed in the presence of NEM or leupeptin. The cleavage activity of BikKam14 in the presence of leupeptin was unaffected (Table 4). No significant increase in substrate cleavage was observed when the gingipain stimulator glycyl-glycine was added to the reaction mixture (Table 4).

TABLE 3 Proteolytic activities of bacterial culture supernatants against *P. gingivalis* substrates[a]

Strain or sample	Proteolytic activity[a]							
	BikKam9	BikKam10	BikKam11	BikKam12	BikKam13	BikKam14	BikKam15	BikKam16
P. gingivalis W50	+++	+++	+++	+++	+++	+++	+++	+++
P. gingivalis K$^-$	+	+	++	++	++	+++	+++	+++
P. gingivalis K1	+	++	+++	+++	+++	+++	+++	+++
P. gingivalis K2	−	+	+	++	++	+++	++	+++
P. gingivalis K3	++	++	+++	+++	+++	+++	+++	+++
P. gingivalis K4	+	+	++	++	++	++	+++	+++
P. gingivalis K5	++	++	++	+++	+++	+++	+++	+++
P. gingivalis K6	+++	+++	+++	+++	+++	+++	+++	++
P. gingivalis K7	++	++	+++	+++	+++	+++	+++	+++
A. naeslundii	−	−	−	−	−	−	−	−
A. odontolyticus	−	−	−	−	−	−	−	−
A. actinomycetemcomitans	−	−	−	−	−	−	−	−
F. nucleatum subsp. *periodontium*	−	−	−	−	−	−	−	−
F. nucleatum subsp. *nucleatum*	−	−	−	−	−	−	−	−
F. nucleatum subsp. *polymorphum*	−	−	−	−	−	−	−	−
P. micros	−	−	−	−	−	−	−	−
P. intermedia	−	−	−	−	−	−	−	−
P. nigrescens	−	−	−	−	−	−	−	−
T. forsythia	−	−	−	−	−	−	+	−
T. denticola	−	−	−	−	−	−	−	−
S. mitis I	−	−	−	−	−	−	−	−
S. oralis	−	−	−	−	−	−	−	−
Saliva	−	−	−	−	−	+	+++	++

[a] Enzyme activity is defined in RF/min values as follows: <5 (−), no activity; 5 to 24 (+), low activity; 25 to 124 (++), moderate activity; >125 (+++), high activity.

Proteolytic activity of *P. gingivalis* gingipain knockout strains. To further explore the role of gingipains in the cleavage of the artificial substrates, the activity of culture supernatants of *P. gingivalis* strains which lack the Arg-gingipains (ΔRgp), Lys-gingipain (ΔKgp), or both (ΔRgp/ΔKgp) was examined. In both ΔRgp and ΔRgp/ΔKgp supernatants, cleavage of the arginine containing substrates was absent (Fig. 1A to E, G, and H). The substrate which consists of only lysine residues, BikKam14, did show cleavage by ΔRgp, though it was with a much lower efficiency compared to the wild-type strain (Fig. 1F). As expected, there was no cleavage of BikKam14 by ΔKgp or ΔRgp/ΔKgp (Fig. 1F). Surprisingly, besides BikKam14, also decreased efficiency of cleavage by ΔKgp was observed on the other substrates (Fig. 1A to E, G, and H).

Sensitivity of the *P. gingivalis* substrates. To study the sensitivity of the *P. gingivalis*-specific protease test, we analyzed serial dilutions of *P. gingivalis* culture using all eight substrates. BikKam9 and BikKam10 showed the lowest sensitivity, with a limit of detection of 10^8 CFU/ml (Fig. 2A). The minimal concentration of *P. gingivalis* that could be detected using BikKam11 to BikKam13 and BikKam15 was 10^7 CFU/ml (Fig. 2A). BikKam14 and BikKam15 showed the best sensitivity, with a limit of detection of 10^6 CFU/ml (Fig. 2A).

To evaluate the possible use of paper points for the collection of clinical samples, paper points were spiked with serial dilutions of *P. gingivalis*. Subsequently, reactivity was tested using the BikKam substrates. It was found that the overall signal was lower than the diluted culture (Fig. 2B). This had a negative effect on the sensitivity of three of the BikKam substrates. The limit of detection of BikKam13 changed from 10^7 CFU/ml to 10^8 CFU/ml and the detection limit of BikKam14 and BikKam16 decreased from 10^6 CFU/ml to 10^7 CFU/ml (Fig. 2B). Despite the decrease in substrate cleavage, the sensitivity of the other substrates remained unaffected.

Screening of patient-derived paper points using *P. gingivalis* substrates. The clinical applicability of the novel protease assay was examined *in situ* by screening 72 paper points obtained from individuals suffering from periodontitis. As a gold standard, the presence of periodontal pathogens was quantified by routine culture (see Table S2 in the supplemental material). Twenty of the paper points were *P. gingivalis* culture positive, varying in *P. gingivalis* concentration from 10^7 to 10^4 CFU/ml. All eluates of the 72 paper points were analyzed using all eight substrates. *P. gingivalis* could be detected to a concentration of 10^6 to 10^7 CFU/ml using BikKam9 and BikKam10 and no false-positive activity was observed (Fig. 3). BikKam11 to BikKam13 showed a somewhat higher sensitivity; however, these substrates were also cleaved by 1 or 2 of the *P. gingivalis* culture-negative samples (Fig. 3). The only L-amino-acid-containing substrates (BikKam14 to BikKam16) yielded the highest sensitivity, but the specificity of these substrates was significant lower than that of BikKam9 to BikKam13 (Fig. 3).

Earlier, Schmidt and coworkers designed an assay for the detection of gingipain R activity based on the use of chromogenic substrates in which enzyme activity was measured using BANA (39). Therefore, the commercially available substrate L-BApNA was included in our paper point study and the results were compared to the results obtained using the BikKam substrates.

TABLE 4 Effects of protease inhibitors and stimulators on the cleavage of FRET substrates by *P. gingivalis* W50[a]

	Relative activity (%)							
Compound	BikKam9	BikKam10	BikKam11	BikKam12	BikKam13	BikKam14	BikKam15	BikKam16
None	100	100	100	100	100	100	100	100
Leupeptin at (μM):								
25	54	48	28	28	29	102	25	24
50	50	42	26	21	24	101	19	15
100	48	42	23	20	21	97	14	11
NEM at (mM):								
6,75	39	27	25	16	14	13	7	4
12,5	30	19	19	11	10	11	4	2
25	23	13	14	8	8	8	3	2
DTT at (mM):								
25	213	165	272	267	235	1,071	364	562
50	275	273	500	564	562	1,570	683	1,164
100	399	340	709	927	886	1,962	1,197	1,722
L-Cysteine at (mM):								
25	213	292	570	701	811	2,728	977	1,920
50	250	334	672	842	920	2,494	952	1,981
100	274	403	849	899	969	2,855	1,027	2,185
Glycyl-glycine at (mM):								
25	98	96	93	99	99	99	96	103
50	97	99	91	99	96	95	89	104
100	96	97	93	94	95	100	92	100

[a] Inhibitors: leupeptin and NEM. Stimulators: DTT, L-cysteine, and glycyl-glycine.

L-BApNA showed a sensitivity for *P. gingivalis* (40%) lower than that of BikKam9 to BikKam13 (60 to 75%) (Table 5). Also the sensitivity of L-BApNA (85%) is lower than that of BikKam9 to BikKam13 (96 to 100%). Overall, the results obtained with our D-amino acid substrates correspond better to the microbiological culture diagnosis. Some of the paper points analyzed contained, besides *P. gingivalis*, other oral pathogens such as *P. micros*, *P. intermedia*, or *F. nucleatum* subsp. *nucleatum*. No significant correlation was observed between the false positives BikKam9 to BikKam13 and the presence of these other oral pathogens (see Table S2 in the supplemental material).

DISCUSSION

The presence of D-amino acids in fluorogenic protease substrates has proven to be crucial for their specificity. Recently we have successfully shown that substrates containing D-amino acids were specifically cleaved by bacterial proteases, as demonstrated for *Bacillus* spp. (20). No cleavage was observed in human secretions or fluids such as saliva or serum (20). To further explore this concept, 115 novel fluorogenic dipeptides were designed, each containing no, one, or two D-amino acids. Screening of this library using culture supernatant of several oral pathogens resulted in the identification of eight *P. gingivalis*-specific substrates. Five of these substrates indeed contained a D-amino acid (Table 2). Almost all of the substrates were cleaved by all of the *P. gingivalis* strains tested, the only exception being BikKam9, where no cleavage activity by *P. gingivalis* capsule serotype K2 was observed. The RF/min value of BikKam9 of this strain was, however, very close to the cutoff value of 5. All three substrates in which no D-amino acid was present (BikKam14 to BikKam16) were also cleaved by proteases present in saliva. Potentially, a diversity of hydrolases present in saliva can be responsible for this cleavage. Cathepsins, for instance, are known to recognize and cleave a wide range of peptide bonds, among which are Arg-Arg, Lys-Lys, and Phe-Arg (6, 10, 15, 22, 30, 32, 42). As well as the cathepsins, other salivary proteases, like alanine aminopeptidase and dipeptidyl peptidase IV, may play a role in the cleavage of these three substrates (2).

In addition to saliva, BikKam15 was also cleaved by the culture supernatant of *T. forsythia*. It is known that *T. forsythia*, similar to *P. gingivalis*, produces secretory proteases (40). One of these proteases, karilysin, has a high preference for substrates which contain a hydrophobic residue, such as phenylalanine, at the P1 position (21).

The sensitivity for *P. gingivalis* of the all-L-amino-acid-containing substrates (BikKam14 to BikKam16) was high. However, due to the presence of D-amino-acid-independent proteolytic activity in saliva, a low specificity was observed (Table 3 and 5). Also, using the clinical samples, a high number of false positives for these substrates were observed (Fig. 3 and Table 5). In parallel to BikKam14 to BikKam16, the commercially available compound L-BApNA also showed a relatively low specificity rate (85%). In contrast, for the D-amino-acid-containing substrates the specificity was high (96 to 100%), strengthening our earlier observation that the use of D-amino acids is crucial in the detection of bacterial proteases using fluorogenic substrates (20).

Despite the increased specificity, the presence of D-amino acids in FRET-peptides had a negative effect on sensitivity. The substrates which consisted exclusively of L-amino acids were able to detect lower *P. gingivalis* concentrations than the D-amino-acid-

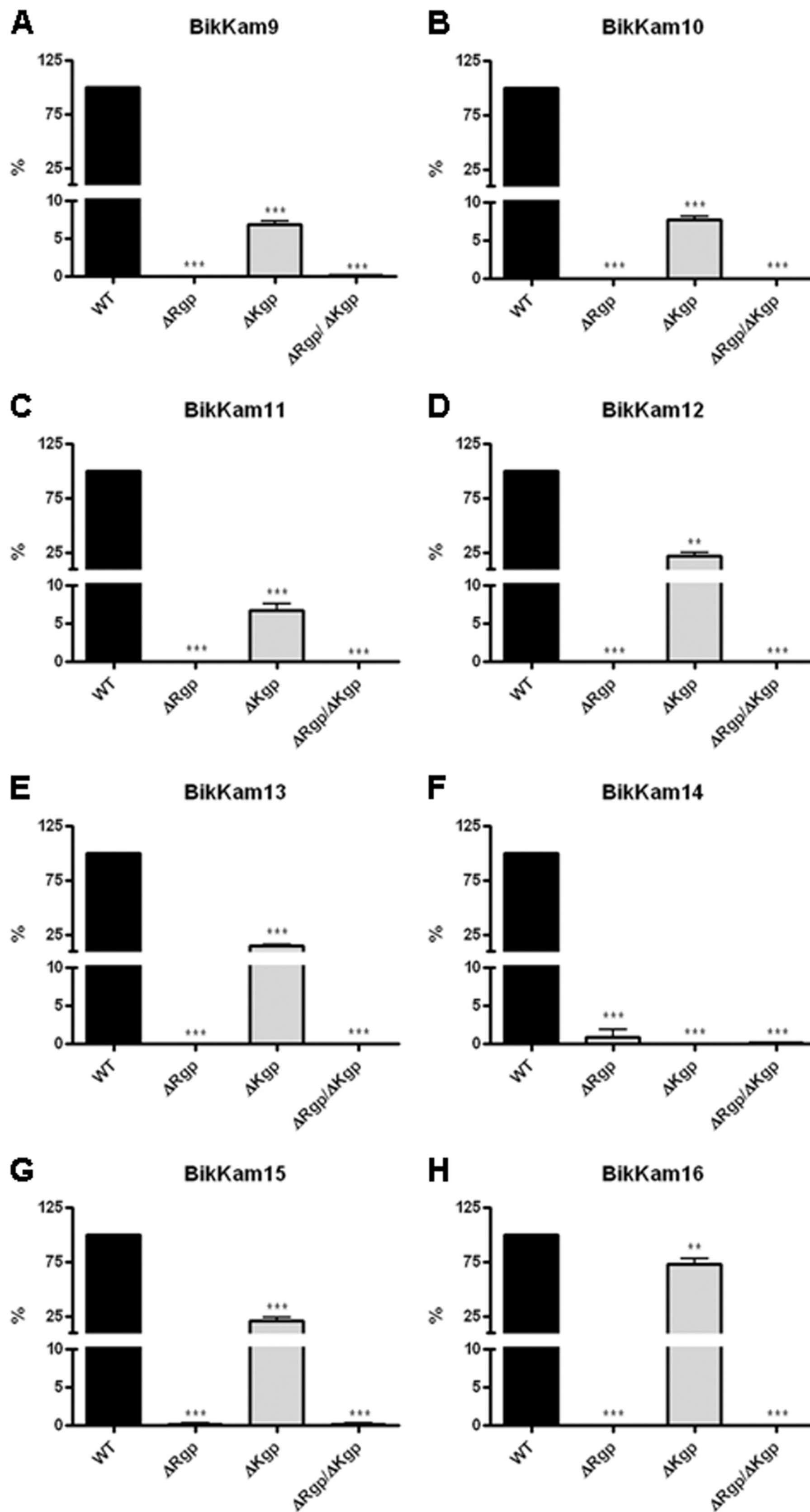

FIG 1 Culture supernatant cleavage activity of gingipain knockout strains. Culture supernatants of *P. gingivalis* W50 knockout strains ΔRgp (KDP133), ΔKgp (K1A), and ΔKgp/ΔRgpA/ΔRgpB (KDP136) were incubated with each FRET substrate (A to H) at 37°C. As a positive control, wild-type strain W50 (WT) was used. Cleavage of the substrates was defined in RF/min. The enzyme activity of knockout strains was compared to *P. gingivalis* W50 activity using the unpaired, two-tailed Student *t* test. Results are expressed as mean ± standard error of the mean ($n = 3$). ***, $P < 0.0001$; **, $P < 0.01$.

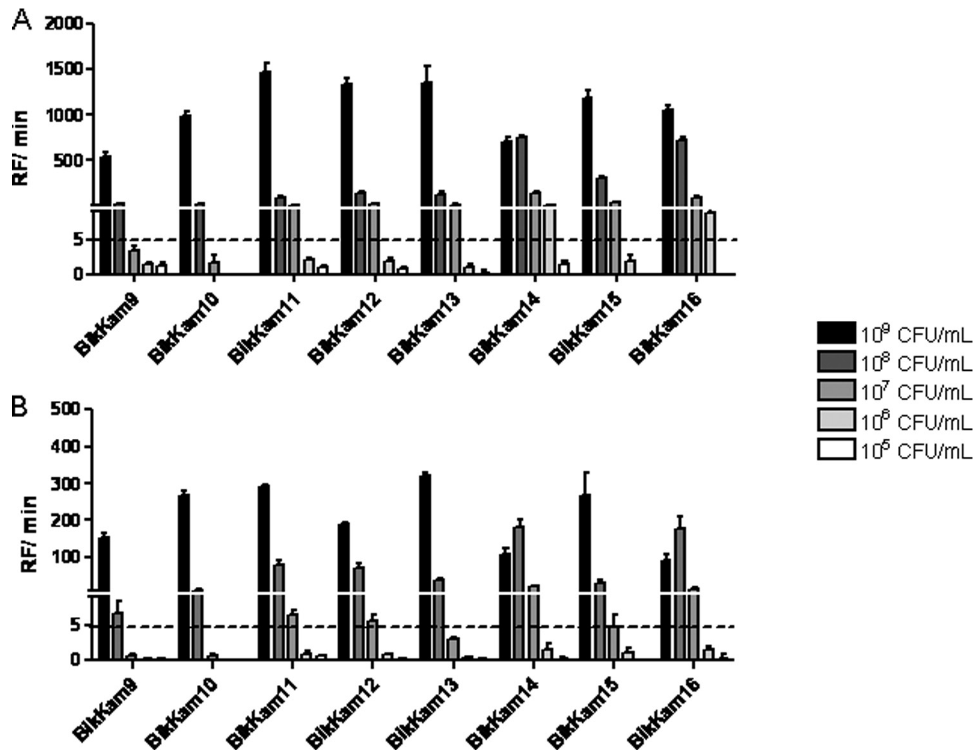

FIG 2 *In vitro* sensitivities of *P. gingivalis* substrates. Serial dilutions of *P. gingivalis* W50 (A) and *P. gingivalis*-spiked paper points (B) were incubated with 16 μM each FRET substrate at 37°C. Enzyme activity was defined in RF/min. The cutoff of the assay was estimated at an RF/min value of 5. Results are expressed as mean ± standard error of the mean ($n = 3$).

containing substrates in culture, as well as in patient samples (Table 3, 5). The sensitivity of the all L-amino acid substrates in diluted *P. gingivalis* culture was 10^6 to 10^7 CFU/ml, whereas for the D-amino-acid-containing substrates, the observed limit of detection was 10 times higher (10^7 to 10^8 CFU/ml). In general, using the patient samples, more efficient cleavage than that obtained with the diluted *P. gingivalis* culture was observed. Compared to culture, the limit of detection for all substrates was 10 times lower (Fig. 2A and Fig. 3). This discrepancy might be due to the fact that the pH of the paper point buffer (pH 7.5) is more optimal for gingipain activity than the pH we measured in our *P. gingivalis* cultures, pH 8.5 (data not shown) (12). Another explanation may be that the amount of gingipains in gingival crevicular fluid is higher, possibly due to the presence of host proteins which might trigger the production of these virulence factors.

Of the 115 substrates from the peptide library, only the substrates which contained an arginine or lysine residue were cleaved by *P. gingivalis* culture supernatants. Therefore, we hypothesized that *P. gingivalis*-specific Arg-gingipains and Lys-gingipain are responsible for substrate cleavage (34).

Previously it had been shown that the proteolytic activity of gingipains can be stimulated by the presence of DTT and L-cysteine and inhibited by NEM (5). The addition of these compounds influenced the proteolysis of all compounds, as expected,

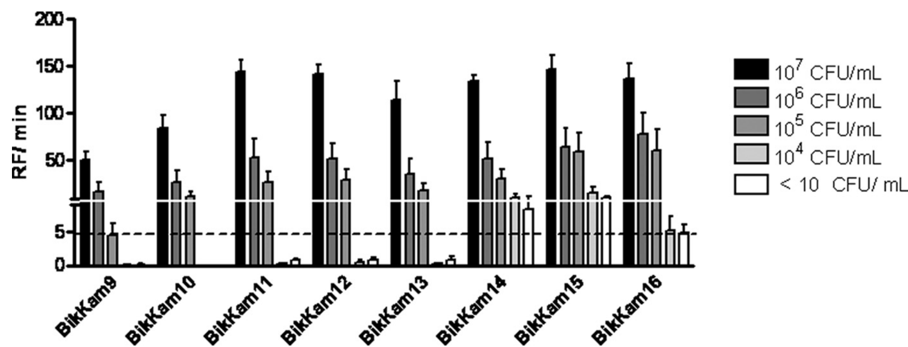

FIG 3 Screening of *P. gingivalis* substrates using patient-derived paper points. A total of 72 patient-derived paper points were incubated with 16 μM each FRET substrate. Fluorescence was measured for 90 min at 37°C. Enzyme activity was defined in RF/min. The cutoff of the assay was estimated at an RF/min value of 5. Results are expressed as mean ± standard error of the mean. 10^7 CFU/ml, $n = 6$; 10^6 CFU/ml, $n = 6$; 10^5 CFU/ml, $n = 5$; 10^4 CFU/ml, $n = 3$; <10 CFU/ml, $n = 52$.

TABLE 5 Validation of *P. gingivalis* substrates using patient-derived paper points

Peptide	Sensitivity[a] (%)	Specificity[b] (%)
BikKam9	60	100
BikKam10	60	100
BikKam11	70	98
BikKam12	75	96
BikKam13	70	96
BikKam14	95	64
BikKam15	95	44
BikKam16	95	73
L-BApNA	40	85

[a] Percent BikKam or L-BApNA positive and culture positive.
[b] Percent BikKam or L-BApNA negative and culture negative.

pointing toward the possible involvement of gingipains (Table 4). It was found that leupeptin inhibited the cleavage of all substrates, except for BikKam14, the only substrate in which no arginine residue is present (Table 4). This finding is in agreement with findings by Aduse-Opoku and coworkers, who observed inhibition of Arg-gingipain activity in the presence of leupeptin, whereas no effect on Lys-gingipain activity was observed (1). A literature search revealed that the amidolytic activity of both Arg- and Lys-gingipain on L-BApNA is stimulated by the addition of glycyl-glycine (5, 7). In the BikKam substrates, however, no amino group is present next to arginine. This can explain the observation that the addition of glycyl-glycine had no effect on the cleavage of our substrates (Table 4).

Experiments using *P. gingivalis* knockout strains further supported the hypothesis that cleavage of the BikKam substrates is mediated by the gingipains. Absence of these gingpains led to significantly reduced proteolytic activity (Fig. 1). In addition to the absence of Arg-gingipain in ΔRgp, it is known that ΔRgp strains secrete Lys-gingipain less efficiently (1, 41). This was confirmed by the decreased proteolytic activity of ΔRgp culture supernatant on the BikKam14 substrate (Fig. 1F). We were surprised to measure a reduced cleavage activity of ΔKgp on the arginine-containing substrates. We hypothesize that since the Dbc group is coupled to the substrate via a C-terminal lysine side chain, cleavage of the BikKam substrates by Lys-gingipain may take place next to the C-terminal KDbc residue. Further experiments are needed to confirm these assumptions. It has to be noted that all peptides present in the library contain a C-terminal KDbc group. Nevertheless, only the eight substrates described in this study were cleaved by *P. gingivalis*. This clearly points toward the essence of the complete amino acid sequence of the substrate for gingipain recognition and proteolysis. The results obtained strongly suggest a role for the gingipains in the degradation of the BikKam substrates. However, the precise chemical basis of BikKam cleavage remains to be elucidated.

The use of an enzyme based diagnostic tool based on the presence of *P. gingivalis* Arg-gingipain was previously described by Loesche and coworkers, who assayed plaque samples using L-BApNA (27, 28). They achieved a sensitivity similar to that of our test using BikKam11 to BikKam13, i.e., $6.6 \times 10^7 \pm 5.8 \times 10^7$ CFU/ml versus 10^7 CFU/ml, respectively. The specificity of the BikKam substrates, however, is significantly higher at 96 to 98% for the BikKams versus 85% for L-BApNA. The L-BApNA assay has been developed into a commercially available strip test which is used as a point-of-care test for *P. gingivalis*, *T. denticola*, and *T. forsythia* (BANA test strips; www.oratec.net). Our results imply that the specificity of this rapid and simple diagnostic tool for the detection of *P. gingivalis* can be improved by the replacement of L-BApNA by one of the D-amino-acid-containing substrates BikKam11, BikKam12, or BikKam13.

Regarding the applicability of these substrates as diagnostic tools for the detection of *P. gingivalis*, their sensitivities, ranging from 10^5 to 10^7 CFU/ml (Fig. 3), potentially match the range in which nonsurgical or surgical therapy is unsuccessful and antimicrobial treatment is needed (35). Consequently, we consider it tempting to hypothesize that the BikKam substrates would not only be helpful in identifying the presence of *P. gingivalis in situ* but also serve as indicators for antibiotic treatment.

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