Activity of pradofloxacin against *Porphyromonas* and *Prevotella* spp. implicated in periodontal disease in dogs – susceptibility test data from a European multi-centre study

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Running Title: Activity of pradofloxacin against agents of periodontal disease

Keywords: fluoroquinolones, pradofloxacin, anaerobes, periodontal disease, dogs
ABSTRACT

Collaborating veterinarians from five European countries collected subgingival bacterial samples from dogs exhibiting clinical periodontal disease. Sterile endodontic paper points were used for collection of samples which were transported to a central laboratory for susceptibility testing. Anaerobic bacteria were isolated and Porphyromonas and Prevotella identified to species level; susceptibility to pradofloxacin and metronidazole, was determined using agar dilution CLSI methodology. A total of 630 isolates, 310 Porphyromonas spp. and 320 Prevotella spp. were isolated. Pradofloxacin MIC data for all isolates was in the range of ≤0.016 to 1 µg/ml, overall MIC₅₀ was 0.062 and MIC₉₀ 0.25 µg/ml. There were no differences in activity against Porphyromonas or Prevotella isolates, nor in the pradofloxacin susceptibility distributions from the different European countries. All isolates were within the wild type distribution and were fully susceptible to pradofloxacin. Metronidazole was also highly active against these strains: 316 of 320 Prevotella strains (98.8%) and 309 of 310 Porphyromonas strains (99.7%) were susceptible (MIC ≤ 8 µg/ml). However, 3 Prevotella strains had intermediate metronidazole susceptibility (MIC = 16 µg/ml) while 1 Prevotella and 1 Porphyromonas strain were metronidazole resistant (MICs of 128 and 256 µg/ml, respectively). Pradofloxacin, a novel third generation fluoroquinolone demonstrates a high degree of anti-anaerobe activity against strains isolated from clinical cases of periodontal disease and shows activity against metronidazole resistant isolates. The broad-spectrum activity of pradofloxacin makes it a suitable candidate for the treatment of periodontal disease in dogs.
INTRODUCTION

Periodontal disease is a chronic, multi-factorial disease of the tissues supporting the teeth (28, 35) and the significance of microorganisms in the development of all types of periodontal disease is indisputable. It is microbial density that is considered critical for the development of gingivitis and some types of chronic periodontitis whilst the type of microorganisms may be of greater importance in the initiation of aggressive periodontitis (41). Indeed, it is now well accepted within the dental research community that periodontal disease results not just from simple accumulation of volume of dental plaque but that the development of the complex plaque leads to growth and dominance of specific pathological organisms. This thinking has arisen from studies of human disease and indeed much of the published data concerning etiology of periodontitis comes from the human arena, where the primary periodontal pathogen is considered to be *P. gingivalis* (7, 35).

Periodontitis in companion animals is an almost identical disease to that in humans in terms of disease course and clinical presentation (17). It has been estimated that approximately 80% of dogs and cats demonstrate some degree of periodontal disease by 4 years of age (19). It is a serious condition that threatens all dogs and is among the most common disorders seen in veterinary medicine (31). The accelerated disease progression observed in companion animals compared to humans may be due to a relative lack of routine dental care (18, 30). Companion animal periodontitis is a serious infection that can have medical consequences such as anorexia and weight loss, chronic pain, sore or loose teeth, swollen gums, tooth decay, breakage or loss of teeth and breakage of the maxillary or mandibular bone (18). If left untreated, periodontal bacteria may spread to other sites in the body via bacteraemia (5, 32) and lead to renal, coronary or hepatic diseases (9, 33, 39). As virtually all cases of periodontal disease are bacterial disorders
they can be prevented or effectively treated by controlling pathogenic microbes residing in subgingival and supragingival plaque (41). In humans and dogs the dental practitioner has relied heavily upon mechanical debridement in combating periodontal infections (18, 41). There is evidence, however, that additional strategies including use of antimicrobials are necessary to effectively combat periodontal infection especially in the case of sites with probing depths exceeding 5 mm (18, 36, 44). For periodontal therapy to be effective it must as a minimum be able to target and effectively control microorganisms capable of destroying periodontal connective tissue. It is well established that the microbial flora associated with periodontitis in humans and dogs is complex (8, 18) and in this context it has been established for a number of years that the absence of the black pigmented anaerobic indicator bacteria such as *P. gingivalis* and *P. intermedia* was a better predictor of no further loss of attachment than the presence of these species was for further disease progression (18). On this basis it has been concluded that antimicrobial therapy can be of great use in the treatment of periodontal disease (38).

**Pradofloxacin** is a third generation fluoroquinolone and like moxifloxacin has enhanced activity against Gram-positive bacteria relative to first and second generation compounds and good activity against anaerobes (40). It has been exclusively developed for use in veterinary medicine although has not yet received regulatory approval, it is distinguished from enrofloxacin, the first veterinary fluoroquinolone, by two structural elements: a bicyclic amine, S,S-pyrrolidino-piperidine, replacing the ethyl-piperazine moiety located at position C-7 of enrofloxacin, and a cyano group which is attached to the C-atom at position 8. The increased potency of pradofloxacin is mainly attributed to the S,S-pyrrolidino-piperidine moiety at C-7, but the cyano group at C-8 extends activity to first and second step FQ-resistant strains. Early data showed its
potential for use against anaerobes (40) and in this paper we report susceptibility data from a
European multi-centre study against strains isolated from cases of periodontal disease in dogs.

MATERIALS AND METHODS
Sample Collection
Canine periodontal pockets were sampled by veterinarians in France, Germany, Italy, Poland,
Sweden and the United Kingdom in the period 2004 to 2006. Each veterinary practice was
provided with sterile endodontic paper points for collection of periodontal pocket samples and
airtight (screw-capped) polypropylene microcentrifuge tubes, containing approximately 1.5 ml of
sterile anaerobic VMGA III medium (46). Tubes were filled in an anaerobic workstation and
minimal headspace was left at the top of each tube prior to despatch.
Participating veterinary surgeons were all experienced with periodontal disease. Dogs suitable for
collection of samples were defined as those showing periodontal disease of grade 2 or grade 3
(moderate to severe periodontal disease). All participating animals had not been pre-treated with
antimicrobials for any disease, by any route of administration, for at least 4 weeks prior to
sampling. It was recommended that samples were to be collected during routine mechanical
scaling or dental surgery while the dog was under general anaesthesia. Only one sample was
collected per tooth, but samples from more than one tooth of the same dog were acceptable as
periodontal disease is considered to be a disease of individual periodontal pockets rather than a
generalised disease (18). However, the maximum number of samples per dog was restricted to
one per quadrant of the dentition giving a maximum of four samples per dog. Samples were
shipped to a central laboratory for isolation, identification and MIC determination of anaerobic
bacteria and were processed within 24 to 48 hours after sampling.
Sample Processing and Identification

Each transport medium containing a paper point was transferred unopened into an anaerobic workstation (Don Whitley Scientific Limited, Shipley, UK) at 35±1°C. All culture media used for sample processing and bacterial subculture were pre-reduced by overnight storage in the anaerobic workstation.

Using sterile forceps, paper points were removed from the transport medium and used to inoculate the surface of Fastidious Anaerobe Agar (LabM, LAB103, Bury, UK, [FAA]) and then placed into Fastidious Anaerobe Broth (LabM, LAB071, Bury, UK, [FAB]). Agar plates and broth were incubated in an anaerobic workstation at 35±1°C for up to 7 days and inspected on each working day. The inclusion of FAB was a precaution, so that the incubated liquid medium would provide an “enriched” sample for recovery of bacteria if no growth was obtained on FAA. In all cases, however, FAA plates yielded growth. Most samples yielded three or more distinct bacterial colony types and from each plate, one colony of each discernible type was subcultured to fresh anaerobic FAA to obtain a pure culture for further identification. Only obligate anaerobes were selected for identification; the specimen collection and transport system allowed for recovery of genera other than Porphyromonas spp. and Prevotella spp. but as the focus of this study was the latter mentioned genera isolation of other anaerobes was not pursued.

Gram-negative rods or coco-bacilli were identified to species level using the Biolog™ system (Hayward, CA, USA) and the Biolog AN MicroPlate™ containing a panel of 95 biochemical tests. These plates were used in conjunction with the Biolog MicroStation system and MicroLog 3 software. The designated quality control strains (Bacteroides fragilis and Porphyromonas gingivalis) were used at all times and Biolog results were only considered valid if correct identification of each control strain was achieved.
Bacterial strains

A total of 310 strains of *Porphyromonas* spp. and 320 *Prevotella* spp. were isolated and identified. Isolates were stored at -80°C in Brain Heart Infusion Broth plus 10% glycerol prior to testing. The *Porphyromonas* strains identified to species level were *P. circumdentaria*/*endodontalis* (126), *P. levii* (49), *P. asaccharolytica* (39), *P. macacae* (39), *P. salivosa* (33) and *P. gingivalis* (24). The *Prevotella* strains were *P. heparinolytica* (77), *P. corporis* (34), *P. nigrescens* (26), *P. oris* (9), *P. disiens* (8), *P. intermedia* (6), *P. oralis* (6), *P. denticola* (4), *P. loescheii* (3), *P. oulorum* (2), *P. buccae* (2) and *P. zoogloeformans* (1). There were a number of *Prevotella* isolates for which species names could not be defined, these were referred to as *Prevotella* spp. (142).

Susceptibility Testing

The test compounds, pradofloxacin and metronidazole, were supplied with a certificate of analysis detailing purity. Minimum inhibitory concentrations (MIC) of both compounds were determined using agar dilution methodology as described by the Clinical and Laboratory Standards Institute (CLSI), formerly National Committee for Clinical Laboratory Standards, in complete accordance with the procedures detailed in M11-A6 using Brucella blood agar (Difco, D0964-17) supplemented with hemin and vitamin K and incubating for up to 48 hours. *Bacteroides fragilis* ATCC 25285 and *Eubacterium lentum* ATCC 43055 were used as quality control organisms. All susceptibility testing was carried out using an automated multi-point inoculator with 25 inoculating pins, under strict anaerobic conditions within an anaerobic workstation (Don Whitley Scientific Limited, Shipley, UK.).
RESULTS AND DISCUSSION

The samples yielded a total of 310 Porphyromonas spp. and 320 Prevotella spp. from cases of periodontal disease. There was good geographic distribution of the isolates, France (26.8%), Poland (23.9%), Germany (18.3%), Sweden (13.7%), Italy (11.3%) and the UK (6%). Within any one country there was an almost equal distribution between the two genera although in Germany Prevotella spp. made up only 41.7% whereas in Italy they predominated (64.8%) relative to the Porphyromonas strains. Clearly both of these bacterial groups are implicated in periodontal disease. It is important to make the point that the identification of the isolates was carried out using the Biolog AN MicroPlate™ system which is unable to distinguish between P. gingivalis and Porphyromonas gulae. P. gulae sp. nov., was proposed as a new species to include strains isolated from the gingival sulcus of animal hosts which were distinct from related strains of P. gingivalis of human origin (11). Irrespective of this finding it is clear from the MIC distribution data (Figure 1) that there are no differences in pradofloxacin susceptibility between these two species.

The summary MIC data is presented by country in Table 1, from which it can be seen that there are no differences in pradofloxacin susceptibility between the different countries. It is for this reason that the overall susceptibility distributions for the two genera are presented in Figure 1 which clearly demonstrates both genera are equally susceptible to pradofloxacin and exhibit the same wild type distribution. On the basis of this distribution there are no strains obviously carrying resistance determinants, the respective populations are clearly fully susceptible to pradofloxacin. MIC data for the respective species of the tested isolates are shown in Table 2. As breakpoints have yet to be assigned to pradofloxacin resistance rates have not been reported although as stated above population distributions suggest an absence of resistance determinants.
Whilst the metronidazole MIC data is similarly consistent by country and for both of the tested genera, strains were identified that were outside the wild type distribution (Figure 2). Three Prevotella strains had intermediate metronidazole susceptibility (MIC = 16 µg/ml) whilst 1 Prevotella and 1 Porphyromonas strain were fully metronidazole resistant (MICs of 128 and 256 µg/ml, respectively). The fully resistant isolates were from Sweden and those of intermediate susceptibility were from Sweden and Poland.

There is clearly a challenge in such multi-centre studies when isolating anaerobes to ensure that the methodology is appropriate and isolation rates are not adversely affected by sampling. VMGA III transport media has been evaluated (3, 8, 46) specifically for anaerobic organisms associated with periodontal disease. It is a gel based transport medium and it is argued that this results in maintenance of a low redox potential thereby contributing to the enhanced survival of obligate anaerobes. The media has been evaluated in clinical studies as well as with pure cultures and has been shown to be suitable for longer periods of transport by mail as necessitated by the logistics of this study. The majority of the received samples (>400) were processed on the day of receipt, 49 samples processed within 24 hours of receipt and only 2 samples were processed 48 hours after receipt in the laboratory. Initial validation work with the transport media had demonstrated that the test organisms remained viable for at least 4 days (unpublished observations).

The availability of anaerobic isolates from periodontal infections in dogs across Europe is extremely limited. This is in part because of the need for specialist facilities and expertise, not just to isolate strict anaerobes but also for their identification. Anaerobes are not routinely cultured within veterinary medicine especially as their presence can be determined generically
from clinical symptoms and treatment is usually empiric. In part this arises because susceptibility test results for anaerobes are often not available for up to five days (24).

Periodontal disease is probably the most common disease in dogs and almost all dogs over five years of age are affected by periodontitis (18). The overall prevalence of periodontal disease has been investigated by several authors and reported as 53% to 97% (4, 12, 15, 16, 20). The frequency and severity of periodontal disease increases significantly with increasing age (12, 16, 20) and decreases significantly with increasing body weight (16, 20). The initial colonisation of the dental pellicle is mainly caused by *Streptococcus* spp. and *Actinomyces* spp. (22). With extension of the supragingival plaque into the gingival sulcus, aerobes consume the available oxygen, thereby creating a low redox potential particularly at the bottom of the gingival sulcus.

These environmental conditions favour the growth of anaerobic organisms. As the disease progresses, deeper periodontal pockets develop with heavy accumulation of bacteria that further lower the oxygen levels. Anaerobes take over and constitute approximately 95% of the subgingival flora in periodontitis (22). During this development, there is also a shift from the predominantly non-motile Gram-positive flora found in the supragingival plaque and the healthy gingival sulcus to a flora of Gram-negative motile anaerobic rods found in periodontal pockets (10). This change can occur within two weeks when plaque is allowed to accumulate (6). The shift of the bacterial flora in periodontitis has been demonstrated by Isogai *et al.* (27) for dogs and is a well known fact in human dentistry. Hence, the isolation of a predominant Gram-negative flora from dental pockets can be viewed as an indicator of periodontal disease. In humans, *Porphyromonas* and *Prevotella* spp. are firmly implicated as periodontal pathogens, and there is an increasing amount of evidence that this is the case in canine periodontal disease (18).

It should be noted here that the canine biotype of *P. gingivalis* should now be referred to as a
different new species *P. gulae* (11). Further new *Porphyromonas* spp. have been isolated from dogs and cats; these include *Porphyromonas (P.) canoris, P. salivosa, P. cangiivalis, P. cansulci, P. crevioricanis* and *P. gingivicanis* (18). In the study of Harvey *et al.* (21), *Porphyromonas* spp. and *Prevotella* spp. were the most frequently isolated anaerobes from subgingival plaque of dogs.

Although periodontal disease can be controlled by mechanical periodontal therapy in the majority of dogs and humans, the literature clearly supports the use of antimicrobial intervention in the treatment of periodontal disease. Slots and Rams (42) concluded that appropriate systemic antimicrobial therapy in human periodontitis enhances clinical outcome in patients with recent or high risk of periodontal breakdown and Nielsen *et al.* (31) demonstrated that in combination with scaling, root planning and polishing, clindamycin dosed at 2.5 mg/lb body weight twice a day for eight days had a significant effect on plaque and pocket depth measures of periodontal disease in dogs but not on gingivitis. Zetner and Thiemann (47) had previously shown that clindamycin given five days in dogs and cats before ultrasonic scaling reduces plaque bacteria by 97.6%. It is clear from the literature that systemic antibiotic therapy, when properly prescribed to patients with aggressive periodontitis can give rise to very good clinical outcome (43) although the value in patients with chronic periodontitis is not as clear. In a recent review Slots and Ting (43) made the point that current periodontal therapy strongly emphasises the suppression or eradication of specific periodontal pathogens yet mechanical debridement may fail to remove pathogens because of their location. The rationale for systemic antimicrobial use is that mechanical debridement may not adequately detoxify the periodontium and that the host immune system may not be capable of eradicating periodontal pathogens (18). Mechanical treatment alone cannot remove invasive bacteria located in the gingiva and the periodontal ligament or bacteria residing
in confined spaces such as the dentinal tubules and the alveolar bone; these bacteria constitute a reservoir for post-prophylaxis re-infection (18).

Single drug therapies with penicillins, tetracyclines, metronidazole or clindamycin have been used frequently in periodontal practice. Since periodontal disease usually harbours a mixture of pathogenic organisms drug combination therapies can be important (43). The need for combination therapies has been to provide the necessary spectrum of activity, not previously available until the recent availability of broad-spectrum fluoroquinolones, active against aerobes and anaerobes.

Whilst much emphasis is made of the role of anaerobes in periodontal disease aerobes should not be ignored. Hennet and Harvey (23) reviewed the role of aerobes in periodontal disease in dogs and on the basis of the mixed aerobic-anaerobic flora commonly found in the early stages of periodontal disease suggested that aerobic bacteria may have an important role in disease development. They showed that as the pathology changes from gingivitis to periodontitis the total number of viable aerobes does not change but the anaerobe/aerobe ratio increases as anaerobes predominate. Studies since then have substantiated the involvement of aerobic Gram-positive organisms, *Staphyloccocus* and *Streptococcus* spp. in gingivitis (21, 47). The aerobes constitute most of the supragingival plaque that causes gingivitis and that subsequently develops into periodontal disease. A reduction of oxygen tension caused by proliferation of the aerobic flora creates favourable growth conditions for the anaerobes (18). In this way it is thought that the aerobic flora plays a crucial role in development of disease. It consequently follows that for antimicrobial therapy to be effective it is advantageous for the active agent to have activity against the anaerobic and aerobic flora. Goldstein (13) emphasised that due to increasing development of resistance of anaerobic bacteria to all antimicrobial agents there is a need to find
new broad-spectrum agents active against both aerobes and anaerobes. Currently available
fluoroquinolones in veterinary medicine only have modest activity against anaerobes. It is clear
that pradofloxacin has enhanced anaerobic activity (40) and further provides broad-spectrum
coverage against aerobic organisms (45).

There is a wealth of literature from the human clinical sector that supports the use of third
generation fluoroquinolones in conditions where anaerobes are implicated (1, 2, 29, 37, 44). The
utility of third generation fluoroquinolones against bacteria associated with dental infections has
also been demonstrated by King et al (26) in a large pan-European study investigating the in vitro
activity of a range of anti-anaerobe antimicrobials against Gram-negative bacilli. Two of the
participating laboratories in this study were indeed dental laboratories, one from the UK and one
from central Europe. The majority of the isolates submitted by these laboratories were P.
intermedia, Porphyromonas spp. and Fusobacterium nucleatum, all isolates implicated in
periodontal disease. Of particular interest are the results for clinafloxacin which is structurally
similar to pradofloxacin and which shows MIC\textsubscript{90} values of 0.06, and 0.125 \(\mu\text{g/ml}\) for P.
intermedia and Porphyromonas spp. respectively.

In conclusion, the literature clearly supports the use of third generation fluoroquinolones for use
against anaerobes. This study is the first report to show that a fluoroquinolone being developed
for use in veterinary medicine has the potential to be used to treat anaerobes implicated in
periodontal disease; this has been substantiated in yet to be published clinical studies.

Pradofloxacin demonstrates a high degree of anti-anaerobe activity against strains isolated from
clinical cases of periodontal disease and shows activity against metronidazole resistant isolates.
The broad-spectrum activity of pradofloxacin makes it a suitable candidate for the treatment of
periodontal disease in dogs.
ACKNOWLEDGEMENTS

This study was funded by Bayer HealthCare AG. Peter Silley has received funds for speaking at symposia organised on behalf of Bayer HealthCare AG and also acts as a consultant to Bayer HealthCare AG.
REFERENCES


Table 1. Susceptibility of oral anaerobes by genus per country and of all strains combined. All summary MIC parameters expressed as µg/ml

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Isolate numbers shown in parentheses
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</tbody>
</table>

NC = not calculated. MIC<sub>50</sub> calculated for n ≥ 4, MIC<sub>90</sub> for n ≥ 10

Table 2. In-vitro activity of pradofloxacin and metronidazole against Porphyromonas and Prevotella spp. isolated from canine periodontal pocket
Figure 1. MIC distribution for pradofloxacin of (a) *Porphyromonas* (310) and (b) *Prevotella* strains (320) isolated from cases of periodontal disease in dogs from a European multi-centre study.
Figure 2. MIC distribution for metronidazole of (a) *Porphyromonas* (310) and (b) *Prevotella* strains (320) isolated from cases of periodontal disease in dogs from a European multi-centre study.