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An improved cost-effective, reproducible method for evaluation of bone loss in a rodent model

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Abstract

Aim: This study was designed to investigate the utility of two ''new'' definitions for assessment of bone loss in a rodent model of periodontitis.

Material and Methods: Eighteen rats were divided into three groups. Group 1 was infected by *Aggregatibacter actinomycetemcomitans* (Aa), group 2 was infected with an Aa leukotoxin knock-out, and group 3 received no Aa (controls). Microbial sampling and antibody titres were determined. Initially, two examiners measured the distance from the cemento-enamel-junction to alveolar bone crest using the three following methods; (1) total area of bone loss by radiograph, (2) linear bone loss by radiograph, (3) a direct visual measurement (DVM) of horizontal bone loss. Two ''new'' definitions were adopted; (1) any site in infected animals showing bone loss >2 standard deviations above the mean seen at that site in control animals was recorded as bone loss, (2) any animal with two or more sites in any quadrant affected by bone loss was considered as diseased.

Results: Using the ''new'' definitions both evaluators independently found that infected animals had significantly more disease than controls (DVM system; $p<0.05$).

Conclusions: The DVM method provides a simple, cost effective, and reproducible method for studying periodontal disease in rodents.

Key words: *A. actinomycetemcomitans*; animal model; bone loss analysis; periodontal disease; rodent

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Rodent models of periodontal disease can provide useful pieces of information regarding host–parasite interactions; however, data derived from these models can, at times, be difficult to interpret (Graves et al. 2007). Specifically, quantitative evaluation of bone loss in experimentally infected animals is complicated by the fact that ''naturally'' occurring bone loss is found in control uninfected animals (Garant & Cho 1979). It is surmized that this background level of bone loss occurs as a result of the interaction of the host animal with its own ''normal'' flora (Klausen 1991). Germ-free rodents, in contrast, have minimal ''naturally'' occurring bone loss but suffer from an ''abnormally'' developed immune system making extrapolation to human disease problematic (Crawford et al. 1978). Specific pathogen-free (SPF) rodents present a good compromise, however the evaluation of bone loss, although improved in SPF rodents as compared with conventional animals, remains an issue (Klausen et al. 1989, Taubman et al. 1989).

Factors such as reduced tooth and jaw size, hair impaction and continued tooth eruption, all can have an impact on interpretation of pathogen induced bone loss in the SPF rodent model (Klausen et al. 1991, Fiehn et al. 1992). To overcome some of these issues both negative control groups (unmanipulated/uninfected) and positive control groups (animals infected with a ''standard'' pathogen to which the animal is known to respond) need to be included in the experimental design

Conflict of interest and source of funding statement

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in order to confirm that levels of bone loss in the experimental group exceeds that found in the normal ''non-manipulated'' group (Chang et al. 1994). In this manner, background or basal bone loss found in ''non-manipulated'' animals can be subtracted from bone loss found in the experimental or intentionally infected animals. The results obtained can then be related to bone loss found in the animals infected with the known standard pathogen. Real differences in bone loss when groups are compared can only be determined if the experimental design includes careful sample size calculations to establish the appropriate number of rodents needed to overcome the natural variation found in each of the animals included in the study groups. Interpretation is further complicated by intra and inter examiner variability in bone assessment which is exaggerated in rodent models due to issues such as tooth and jaw size, etc. as described above.

As a result of the complications described above, interpretation of data comparing ''naturally'' occurring bone loss in control animals to bone loss resulting from infection with specific periodontal pathogens in a rodent model of periodontal disease has been fraught with difficulties (Graves et al. 2007). While animal models can provide useful longitudinal data related to pathogenic events in periodontal disease, investigators are often discouraged from attempting to use these models because of the number of animals required, cost of animal upkeep, labour intensive efforts, and the known variability in interpretation reported in the literature (Klausen 1991). Thus the goal of this study was to determine the utility of defining "true" bone loss on a site-by-site basis in experimental animals as compared to their controls. Subsequently, the number of sites experiencing "true" bone loss was then used to diagnose disease in a rodent model.

This paper describes a method that overcomes many of the difficulties described above by defining ''true'' bone loss as loss of bone at a particular site in an experimental animal that is greater than two standard deviations (SDs) above the mean bone loss at that same site in the control uninfected animals. Further ''real'' periodontal disease is defined as any animal in the experimental group with bone loss, as described above, in two or more sites in any one quadrant.

Material and Methods

Creation of leukotoxin (Ltx) mutant strain of *agrgregatibacter actinomycetemcomitans*

The polymerase chain reaction was used to amplify the Ltx gene in *A. actinomycetemcomitans* (Balashova et al. 2006). The amplified DNA was ligated into a cloning vector, transformed into an *Escherichia coli* host, and the plasmid was mutagenized using an in-vitro transposon mutagenesis kit (EZ::TN epicentre). Once the plasmid clone was identified, directed mutagenesis was carried out in-vivo in strain DF 2200N/rifampin (Rif) [a spontaneous naladixic acid (N) and Rif resistant variant of DF 2200] as described (Bhattacharjee et al. 2007). Recombination by allelic exchange was selected for in DF2200N on AAGM containing kanamycin (40 ug/ml) (Balashova et al. 2006).

Bacterial strains, culture conditions and maintenance

All strains were derived from *A. actinomycetemcomitans* DF 2200, a serotype ''a'' strain with a 652 Ltx type promoter, isolated from a patient with aggressive periodontitis and maintained in its rough, adherent form on AAGM agar (Fine et al. 1999). Strains were grown in 100 ml of AAGM medium containing 35 ug/ml of Rif in tissue culture flasks in an atmosphere of 10% CO2/90% air for 2–3 days at 37°C. *A. actinomycetemcomitans* cells that adhered to the wall of the flask were removed by scraping. Cells were re-suspended in phosphate-buffered saline (PBS) containing 3% sucrose, vortexed and resuspended to an approximate optical density of OD 560 = 0.8 equivalent to 108 cells/ml.

Animal models

Eighteen SPF Sprague-Dawley male rats (5–10 weeks of age) weighing between 150 and 250 g were purchased from Taconic farms (Taconic, NY, USA), housed in separate cages, and fed powdered food (Laboratory Rodent Meal Diet 5001, Purina Mills Feeds, St. Louis, MO, USA). Rats were given 20 mg/ml Kanamycin and 20 mg/ml Ampicillin in their drinking water for 4 days to depress the resident ''natural'' microbial flora. Rat's mouths were also swabbed with chlorhexidine gluconate 0.12% for 2 additional days. After a wash-out period of 3 days the rats were divided into three groups of six rats each (Schreiner et al. 2003). Before inoculation, animals in each group were bled via their tail vein to establish initial pre-inoculation serum titers to *A. actinomycetemcomitans*. Each experimental group received the following food: Group 1: received food containing wild-type *A. actinomycetemcomitans* DF2200 N, Group 2 received food with strain DF 2200 N containing the Ltx A mutant strain, while Group 3 received animal feed with no bacteria added.

Feeding of animals

After fasting for 3 h, Groups 1 and 2 were provided with 1 g of powdered food placed in special feeder trays containing 10^8 *A. actinomycetemcomitans* cells in 3% sucrose in PBS. Group 3 received food containing 3% sucrose in PBS but devoid of any bacterial inoculum. After 1 h the inoculated food was removed and replaced with regular powdered food. The inoculation-feeding regimen was repeated for 8 days (Schreiner et al. 2003).

Sampling of the rat oral flora

All sampling was done after rats were anaesthetized intraperitoneally (i.p.) with 40–60 mg/kg of ketamine and 1–2 mg/kg of acepromazine. Following the last inoculation, rats were placed on a diet of regular powdered food. Two weeks later the rats were anaesthetized and their oral flora was sampled with a cotton tip swab for soft tissue sampling, and with a balsa wood toothpick, i.e., a Stimudent (Johnson & Johnson, Piscataway, NJ, USA) for hard tissue sampling. Samples were placed in individual tubes containing 1 ml of PBS. Ten weeks later the final sampling was performed (Schreiner et al. 2003).

Determination of Aa colonization

One hundred microlitres of the collected sample obtained from soft and hard tissue surfaces in individual rats were subjected to serial dilutions in PBS. Dilutions were plated on Trypicase soy agar containing 10% sheep's blood for total anaerobic counts, and on AAGM and AAGM containing Rif at a concentration of 35 mg/ml for quantitation *A. actinomycetemcomitans* and its Ltx mutant. For total counts, plates were incubated in an anaerobic chamber (Coy, Grasslake, MI, USA) at 37°C for

5–7 days. *A. actinomycetemcomitans* selective plates were incubated for 2–3 days in an atmosphere of 10% CO_2 at 37°C. Plates were evaluated for number of colony forming units/ml. Counts were determined in a blind manner for all samples for each rat in each group at both the 2- and 12-week period after feeding.

A. actinomycetemcomitans antibody assay

The enzyme-linked immunosorbent assay was used to determine IgG antibody to *A. actinomycetemcomitans* as described previously (Schreiner et al. 2003). Serum derived from initial tail vein bleeds before bacterial feeding was compared with blood obtained by cardiac puncture.

Bleedings were stored at −70°C. *A. actinomycetemcomitans* lysates were prepared from 3-day cultures (adjusted to 10^8 bacteria/ml = OD_{560} 0.8) re-suspended in 1 ml PBS and then re-suspended in 1.2 ml TEN buffer (Schreiner et al. 2003). Background levels of antibody that cross-reacted with *A. actinomycetemcomitans* were determined by comparison to control rat sera and pre-immune sera. All assays were run in duplicate (Schreiner et al. 2003).

Analysis of bone loss

After the 12-week sampling, rats were euthanized with sodium phentobarbital (100 mg/kg i.p.), subjected to cardiac puncture (blood for antibody analysis) and their heads were removed and stored at −70°C. Rat maxillae were cleaned and de-fleshed by autoclaving for 10 min. in preparation for analysis of bone loss. Three methods were used as follows; (1) a radiographic method that measures the total amount of interproximal bone loss [total area measurement (TAM)] (Schreiner et al. 2003), (2) a radiographic method that measures bone loss from the cemento-enamel-junction (CEJ) to the alveolar bone crest (ABC) [linear measurement (LM)] (Klausen et al. 1991), and (3) a direct visual measurement (DVM) by digital photography that measures bone loss from the CEJ to the ABC using a methylene blue stain and visual assessments [digital visual method of measurement (DVM)] (Chang et al. 1994). Two examiners were trained in each method. Before analysis of bone loss a calibration exercise was performed (see below after description of bone loss methods).

Radiographic method: TAM

Radiographs were taken using Kodak INSIGHT film at setting of 90 KV, 15 ma, using a 3/60 s exposure. A soft wax mount was used to orient bones perpendicular to the X-ray source. The X-ray emitter cone was placed flush to the table to ensure that the distance from the emitter to maxillae was constant. To avoid scorer bias, radiographs were assigned a random code number. Radiographs were projected against a white wall to produce a × 6.25's magnified image (Schreiner et al. 2003). Briefly, to measure total bone loss, the area between the CEJ and the ABC surrounding the first, second and third molars were measured, as were the areas of bone between furcations between the roots. These areas were traced from the projected images. Tracings were scanned into digital files with a Microtek Scan Maker III (Microtek, Taiwan, ROC), and the area of bone loss was calculated using Canvas (Deneba Software, Miami, FL, USA). Two independent investigators evaluated bone loss. Each evaluator performed a site-by-site correlation analysis (Fig. 1a).

Radiographic method: LM

Vertical bone loss was evaluated by radiograph using a modification of the LM method described by Klausen et al. (1991). Each coded radiograph was scanned using a CanoScan LiDE 500F scanner (Canon Inc., Lake Success, NY, USA) and the scans were printed by an Epson Stylus Photo R320 (Epson America Inc., Long Beach, CA, USA) on 4 × 6 glossy paper (Kodak Ultima Picture Paper, Eastman Kodak Co., Rochester, NY, USA). The print was enlarged fourfold. The vertical distance between the CEJ and the ABC were measured at six points adjacent to the three maxillary molars on both sides of the jaw (sites A, C D, F, G and K.; Fig. 1b). Tooth furcations were measured from the top of the furcation to the bone crest (sites B, E H and J; Fig. 1b). The distance was measured in mm by putting a pencil-mark on the CEJ (or furcation) and ABC and then measuring the distance between the two points with an electronic digital calliper (Marathon, Ontario, Canada).

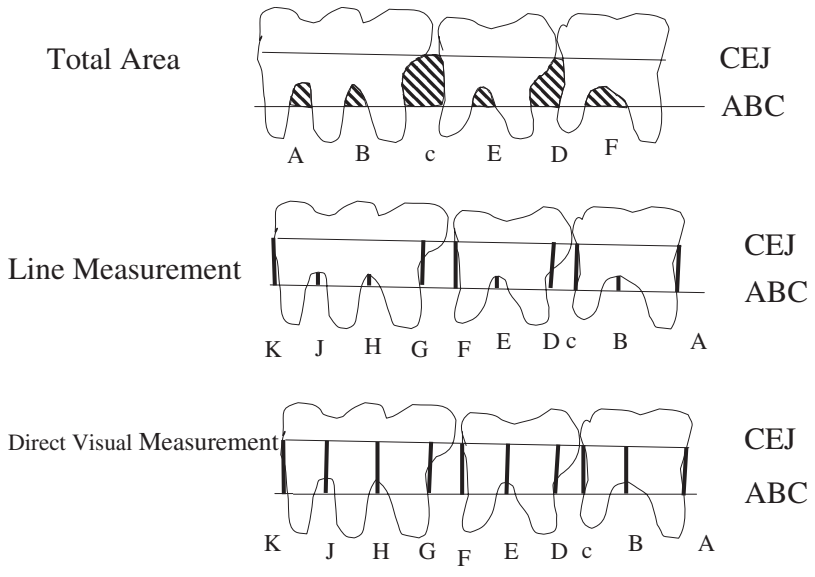

Fig. 1. Illustration of three methods of bone loss evaluation. Top diagram represents the total area measurement (TAM) method using radiographic analysis. The total area is traced, projected and calculated for each of six points indicated between the three molar teeth. Middle diagram represents the line measurement (LM) method using radiographic analysis. Distance from the cemento-enamel-junction (CEJ) to alveolar bone crest (ABC) is indicated by a pencil mark which is then calculated for each of 10 point indicated for each of the three molar teeth. Bottom diagram represents the direct visual method (DVM) of measurement of bone loss using digital photographs taken using a camera attachéd to a dissecting microscope set at low magnification. Distance from the CEJ to ABC is measured for each of 10 points indicated for each of the three molars.

Photographic method: DVM

The maxillae were stained with 1% methylene blue so that the CEJ could be readily distinguished. The jaws were positioned with the lingual side facing up so that the occlusal plane of the molars was perpendicular to the microscope stage. An Oympus stereoscopic microscope was used to examine the images. Photographs were taken of the stained jaws using a DP12 microscope digital camera (Olympus, Center Valley, PA, USA) at $\times 9.2$'s magnification. The DVM was accomplished by a modification of the method by (Chang et al. 1994). The photos were printed on an Epson Stylus Photo R320 (Epson America Inc.) on 4×6 glosssy paper (Kodak Ultima Picture Paper, Eastman Kodak Co.) and coded so the examiners were unaware of the treatment group to which the jaw belonged. The vertical distance between the CEJ and the ABC were measured at 10 sites (sites A, B, C, D, E, F, G, H, J and K.; Fig. 1c) on both right and left maxillary jaws. For improved precision, the distance was measured in using an electronic digital caliper (Marathon).

Calibration

The two examiners went through a rigorous training program and a calibration exercise for each of the three methods. Training and calibration consisted of evaluation of X-rays for bone loss using the TAM and LM methods, while photographs were used for evaluation of bone loss in the case of the DVM. All sites in each of the three groups of animals were examined. The two examiners (H. S., a microbiologist, and C. N.-H. a dentist) were required to perform repeat examinations on a series of randomly selected and coded X-rays and photographs and intra and inter examiner correlations were determined. The two examiners were chosen because they had little experience performing clinical evaluations or assessing rodent radiographs and would therefore serve as good candidates for evaluation of the methods proposed. The random code was computer generated and the same sequence was followed by each examiner. Examiners were blinded to the code. For the calibration exercise a correlation coefficient of $r = 0.7$ was considered acceptable (see Table 1).

Table 1. Correlation of readings of bone loss within and between examiners

Method	Examiner 1	Examiner 2
1. Intra-examiner correlation		
TAM	0.85	0.85
LM	0.88	0.78
DVM	0.907	0.801
Method	**Read 1**	**Read 2**
2. Inter-examiner correlation		
TAM	0.87	0.86
LM	0.788	0.758
DVM	0.755	0.796

TAM, total area measurement; DVM, direct visual method ; LM, line measurement.

Statistical analysis of bone loss, antibody titres and colonizaton

Following the successful completion of the calibration exercise, bone loss was evaluated in each of the groups using the three methods. Each examiner performed two independent readings. An ANOVA was performed to determine if statistically different amounts of bone loss per site occurred in the animals fed the wild type or ltx deletion mutant strains as compared with the control animals that were not fed Aa. For statistical significance to be achieved a p value of <0.05 was required. Each reading by each examiner was analysed independently. Because there were no major differences seen in the two separate readings, the readings were combined and their means presented. Based on prior experiments a sample size of six animals/group was deemed adequate to detect statistically significant differences between groups with 80% power (Schreiner et al. 2003). However, we soon realized that use of strain *A. actinomycetemcomitans* DF 2200 produced 40–50% less bone loss in the experimental animals as compared with that seen in our original experiments that used strain CU 1000 (Schreiner et al. 2003). Bone loss from our original experiments provided the basis for our sample size calculations. Nevertheless, bone loss in the control group in this study and previous studies were similar. These comparisons suggested that the study design was adequate but that the methodology was not sensitive enough to detect the more subtle differences in bone loss in the experimental animals challenged with this less pathogenic *A. actinomycetemcomitans* strain DF 2200. As a result, two other standard methods, the LM and DVM methods were used to compare bone loss in control and experimental animal groups.

In another comparison between the two experiments (initial and current), antibody titre levels and colonization levels in animals that demonstrated bone loss were compared with titres and colonization levels seen in controls. Elevated antibody titres and colonization were analysed by determining whether these values were above background using a simple yes or no response; yes, they did show antibody titres or colonization above background seen in controls, or, no the animal did not reach a level above background. This data was evaluated by a χ^2 analysis and to achieve significance a p value <0.05 was required and elevated antibody titers and colonization were statistically elevated in the experimental animals challenged with both CU 1000 and DF 2200.

With this data at hand we were convinced that the methodology used to evaluate bone loss needed revision. As such it was decided to incorporate two ''new'' principles into the definition of bone loss and disease in efforts to reduce background noise and to improve the sensitivity and specificity related to evaluation of bone loss. Thus, for a particular site in an infected animal to show ''*true*'' bone loss that site was required to show a level of bone loss that was greater than two SDs above the mean bone loss at that same site in the control animals. Further, for an animal to be diagnosed with ''*real*'' disease that animal was required to have ''*true*'' bone loss at two or more sites in any one quadrant. Moreover, using this definition of ''*true*'' bone loss and ''*real*'' disease, we repeated the calibration exercise by selecting a sample of X-rays and photographs to make inter and intra examiner comparisons using each of the three methods of evaluation. Each evaluator performed two separate examinations. A κ statistic was calculated using this new definition of disease for both the inter and intra examiner scores for the infected (experimental) and non-infected (control) animals. To achieve success a κ score of 0.7 was required (see Table 3).

Following calculation of κ, ''true'' bone loss and ''real'' disease were analysed and the wild type infected and control animals were compared. Once again repeat measures were performed

by each of the two evaluators using each of the three measurement systems (TAM, LM and DVM). Statistical significance at the $p < 0.05$ was required as determined by the Fischer exact test (Table 4).

Results

Table 1 shows the correlation coefficient of the inter-examiner and intra-examiner repeat measurements comparing the two examiners and the three systems of measurement in the preliminary evaluations. Results indicate that both inter and intra examiner readings fit within the parameters ($r = 0.7$ and above) of the system that we used initially to evaluate repeatability of readings. A correlation coefficient of $r = 0.85$ was attained when the two examiners were compared; while an $r = 0.87$ or $r = 0.86$ was attained when repeat readings were done by the same examiner.

Figure 2 illustrates an example of results obtained in bone loss measurements made in experiments used to calculate sample size using the TAM system. In this figure the total bone area measurements are depicted. We chose to depict the scores obtained from one examiner; however, numerical scores from both examiners can be seen in Table 2. As seen in Fig. 2 more bone loss was found in the group fed DF 2200, the wild type strain of *A. actinomycetemcomitans*. However, differences between the rats in wild type fed group and Ltx mutant strain fed group and control fed group were not significant. The mean for bone loss derived from the group fed the wild type *A. actinomycetemcomitans* was greater although not significantly so as compared with the mean for bone loss obtained from the control non-infected group or the Ltx mutant group (wild type = 0.66+0.07; mutant fed = 0.57+0.16; control group = 0.58+0.13; $p = 0.43$). Table 2 is a more detailed presentation of the data and shows the mean data derived from measurements of each of the two examiners in the preliminary studies used for sample size calculations. Bone loss was compared in the uninfected and infected animal groups using the three standard methods of evaluation (TAM, LM and, the DVM using ANOVA to analyse bone loss measurements. The table presents the mean data obtained for the two scores for each of the two examiners for each of the three systems of measurement. In the case of the DVM method, a statistically significant level was found by one examiner at one of the measurement periods. No other measurement achieved statistical significance.

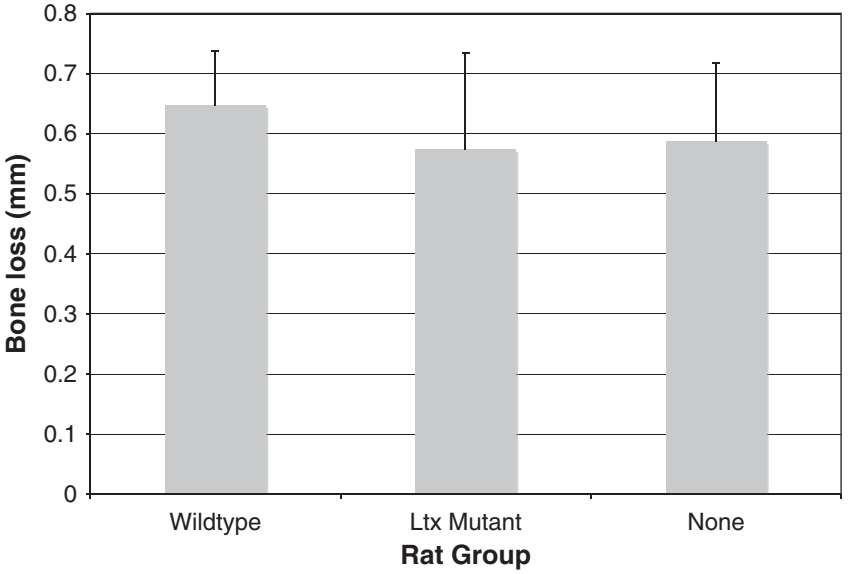

Fig. 2. Level of bone loss for the experimental and control groups. The total area measurement was used to determine the amount of bone loss for each of three groups studied.

Table 2. Evaluation of total bone loss in three treatment groups*

Group	Examiner 1			Group	Examiner 2		
	total bone loss per rat				total bone loss per rat		
	mean	SD	*F* value *p* value		mean	SD	*F* value *p* value
Total area measurement (TAM)							
Wild type	0.66	0.07	$F = 0.90$	Wildtype	0.43	0.06	$F = 3.50$
Ikt mutant	0.57	0.16	$p = 0.43$	Ikt mutant	0.29	0.11	$p = 0.06$
None	0.58	0.13		None	0.33	0.11	
Line measurement (LM)							
Wild type	35.54	5.7	$F = 0.32$	Wildtype	25.34	6.98	$F = 1.53$
Ikt mutant	32.81	8.75	$p = 0.73$	Ikt mutant	19.49	6.38	$p = 0.25$
None	32.75	4.77		None	19.04	7.22	
Direct visual measurement (DVM)							
Wild type	119.01	8.85	$F = 3.90$	Wildtype	111.21	9.02	$F = 2.67$
Ikt mutant	106.97	7.13	$\mathbf{p = 0.04}^{\dagger}$	Ikt mutant	103.13	10.47	$p = 0.10$
None	98.38	19.31		None	92.06	20.46	

*Three treatment groups = A40 *A. actinomycetemcomitans* (Aa) infected (Aa wild type), Aa Ikt mutant infected, non-infected (none).

†Using ANOVA and Duncan's grouping, the mean of the wildtype group is significantly different from the mean of the none group and the Ikt mutant group.

TAM, total area measurement; DVM, direct visual method; LM, line measurement.

Two new rules were adopted; one for ''*true*'' bone loss and one for ''*real*'' disease. Using these new definitions a κ statistic was performed to determine examiner repeatability. The results from the κ statistic as seen in Table 3 represent the measure for ''*real*'' disease in any particular animal (keeping in mind that bone loss had to achieve a level of two SDs or more above the mean bone loss at that site in the control animals and real disease required two or more sites in at least one quadrant in any animal that showed ''true'' bone loss). Using this ''new'' system of analysis, repeat measures by the either examiner (intra examiner evaluations) using TAM failed to achieve any agreement. In contrast, when the new definitions are used the LM appeared to achieve reasonable agreement within examiners.

Table 3A. κ statistic: intra- and inter-examiner agreement, defining two sites or more as diseased

	Intra-examiner κ		
	Examiner 1	Examiner 2	Examiner 1+2
Between TAM 1a and 1b	−0.06	0.3	0.07
Between LM 2a and 2b	**0.87**	**0.48**	**0.67**
Between DVM 3a and 3b	**0.88**	**0.63**	**0.75**

Table 3B. κ statistic: measure of agreement when examiners are compared (inter-examiner κ)

	TAM1a	TAM1b	LM2a	LM2b	DVM3a	DVM3b
Between examiner 1 and 3	0.19	-0.22	0.33	**0.48**	0.38	**0.88**

κ levels significantly different from zero are in bold type.
TAM, total area measurement; DVM, direct visual method; LM, line measurement.
a = first read
b = second read

Table 4. Comparison of disease in control animals *versus* experimentals; evaluation of two examiner scores using three methods

Treatment	Examiner 1 *p* values*		Examiner 2 *p* values	
	read 1	read 2	read 1	read 2
(A) Total area measurement				
None/wt	0.3487	**0.0362****	0.075	0.6105
None/Ikt mutant	0.6479	0.1254	1	0.6105
(B) Line measurement				
None/wt	0.2354	0.1042	**0.0480****	**0.0480****
None/Ikt mutant	0.1042	0.1042	0.2986	0.2986
(C) Direct visual measurement				
None/wt	**0.0083****	**0.0083****	**0.0453****	**0.0104**
None/Ikt mutant	0.2355	0.4542	0.2769	0.2769

*p values represent the result of pairwise comparisons of numbers of diseased animals in treatment groups using Fisher's exact test and the Bonferroni correction.
**Significant difference between number of diseased animals in the treatment groups.
The raw data for bone loss was assessed using the three methods: (A) area, (B) line measurement and (C) direct visual measurement. Diseased animals were defined as rats having two sites of ''real'' bone loss per quadrant.
TAM, total area measurement; DVM, direct visual method; LM, line measurement.
Reading showing significant differences beta groups area in bold typo.

However, when overall intra-examiner evaluations of the three methods were compared, the DVM method appeared to achieve the best agreement. This advantage becomes more evident when inter-examiner comparisons were evaluated. As seen in the table, since κ scores of 0.7 and above were considered acceptable, only the DVM score achieved that level when the evaluators were compared (Table 3). However, it is worth noting that a learning curve was required even in the case of the simplified DVM method (compare first read to second read Table 3b).

When the new definition of ''*real*'' disease was used only the DVM measure provided consistent results (Table 4). In the case of the LM, scores of examiner 2 achieved statistical significance when the control animals were compared to wild type animal scores. In comparison, scores by examiner 1 did not show these differences. In the case of the TAM, examiner 1 found differences in scores obtained from the wild type and control animals while examiner 2 did not.

Discussion

In previous experiments our group developed an animal model in which healthy ''noninfected'' rodents were fed *A. actinomycetemcomitans* to induce colonization, an immune response and bone loss (Schreiner et al. 2003). Analysis of bone loss was accomplished by radiographic analysis (Schreiner et al. 2003). In that experiment the addition of *A. actinomycetemcomitans* strain CU 1000 to the animal feed produced significant increases in bone loss as compared with non-fed control animals. Since the addition of *A. actinomycetemcomitans* to the animal feed was the only variable in the experimental design we felt that it was logical to deduce that increases in bone loss could be attributed to *A. actinomycetemcomitans*. These results encouraged us to examine particular virulence genes possessed by *A. actinomycetemcomitans* that could be responsible for the potential to initiate the bone loss we observed. However, CU 1000 was resistant to genetic manipulation and thus we chose to use DF 2200 as the host for gene inactivation. Subsequently, we found that wild-type DF 2200 produced 40–50% less bone loss as compared to wild-type CU 1000 using the same experimental protocols as was used in the earlier experiments. On the other hand, the uninfected control groups from both DF 2200 and CU 1000 experiments when compared showed similar amounts of bone loss. Furthermore, both antibody titers to *A. actinomycetemcomitans* and colonization by the microbe were statistically elevated in the animals fed either CU 1000 and/or DF 2200 as compared with control animals.

In conclusion, data obtained from two independent experiments indicated that CU 1000 and/or DF 2200 fed animals had; (1) statistically elevated antibody titres and levels of *A. actinomycetemcomitans* colonization, (2) basal bone loss that was similar in un-infected control groups in both CU 1000 and DF 2200 infection models, and (3) bone loss in DF 2200 wild type fed group was greater, although not statistically greater, as compared to the control non-infected group as compared with CU 1000 infected animals where bone loss was significantly greater than controls. As a result, we were led to believe that when the DF 2200 infection model was used, the system of bone loss measurement lacked the sensitivity and specificity required to distinguish between *DF 2200*-induced bone loss and naturally occurring bone loss. We then sought to examine different methods of bone level measurements in rodents to determine whether measurement of bone loss could be improved.

Three common methods of evaluating alveolar bone loss in rodents have been reported and were used for comparison (Chang et al. 1994). In addition to comparing these three methods we

decided to adopt two ''new'' principles for evaluation of bone loss. The first principal was based on the fact that clinical studies of human periodontal disease utilize a method of measurement that stipulates that a ''true'' measure of attachment loss at any particular site occurs when probing for a change in clinical attachment, shows a minimum of 2 mm of attachment loss above that seen at the last measurement recorded at that site. Thus any measure that shows attachment loss that is <2 mm above the mean recorded at the previous visit may not signify ''real'' change at that site (Goodson et al. 1984, Goodson 1986). This rule has been applied to reduce background noise or measurement error. A similar principle was applied to our animal model and thus ''true'' change was defined as any site that showed bone loss that was more than two SDs above the mean bone loss seen at that site in the control un-inoculated animals.

The second principle that we adopted required that we take into account the fact that rodents lick themselves which impacts hair in-between molar teeth, creating traps for plaque which most likely accounts for bone loss in SPF tooth sites in ''noninfected/non-inoculated'' animals (Klausen 1991). We estimated that hair impaction on average would account for excessive bone loss in one tooth-site per quadrant per animal. Thus in adopting the second principle we decided to define ''real'' disease for a particular animal, as any animal where two or more molar tooth sites had bone loss (>2 SD above the mean loss at that site in the control) in a given quadrant. In a recent publication Wilensky et al. (2005) demonstrated the clear advantage of using micro-computed tomography for bone level evaluation in a mouse model of periodontal disease (Wilensky et al. 2005). Researchers were able to show highly significant differences in bone loss in the six animals challenged by *Porphyromonas gingivalis* as compared with six control animals ($p<0.001$). However, no difference was seen when comparisons were made using the traditional morphometric (linear) techniques (our LM method). The researchers concluded that this sophisticated method of micro-computed tomography allowed investigators to obtain data required to test a well-defined hypothesis while still using a minimum number of animals in each group. However, while this method is technologically sophisticated it remains cost-prohibitive. When we considered the above data (Wilensky et al. 2005) and the data we obtained in this recent study using conventional techniques of bone level analysis, it was our feeling that an improved more cost-effective method for evaluation of bone loss was warranted. The problems of bone loss assessment in rats were reminiscent of problems addressed 20 years ago when measurement of periodontal disease in humans was redefined (Goodson 1986). As such we adopted two ''new'' principles for evaluation of bone loss in rodents that paralleled principals devised for evaluation of periodontal disease in humans. By adopting these two new definitions we were able to repeat our calibration exercise and the inter and intra examiner comparisons that followed. Adopting these new principles enabled the DVM method to produce reliable data that showed agreement for two independent investigators. It is possible that the direct visual method improves reproducibility because detection of the CEJ is simplified when roots are stained and direct measurements are compared with CEJ detection by radiograph and that this better visualization reduces the scorer variability. While it is our belief that these ''new'' methods present a significant improvement in analysis of bone loss, sequential analysis is required to gain a better understanding of pathogenic effects on the host. To overcome the fact that sequential analysis cannot be done on the same animal we are currently performing experiments where animals are sacrificed at different time intervals and then undergoing both clinical and histological evaluation to further clarify these important time dependent events. In spite of the lack of sequential analysis in this study we feel confident from our results that since Ltx is specific for primates and humans it does not play a significant role in the pathogenesis of periodontal disease in this rat model.

The authors are hopeful that the definitions developed can be useful to researchers who study periodontal disease in rodents. In our hands the method was relatively easy to learn (a learning curve of two rounds of reading is required), demonstrated reduced variability in scoring and was cost-effective since fewer animals were required to achieve statistical power. However, additional studies by others are warranted to corroborate the utility of the definitions we have suggested to reduce scorer variability. For our purposes, our immediate goal is to use this improved system to evaluate microbial variants and rodent strain differences that lead to increased bone loss. Our long-term goals are; (1) to identify *A. actinomycetemcomitans* genes responsible for the pathological events seen in *A. actinomycetemcomitans* – induced bone loss, and (2) to examine genetically different rat strains in order to gain a better understanding of periodontal pathogenesis.

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Clinical Relevance

Scientific rationale for the study: Animal models can supply information regarding the pathogenesis of periodontal disease. However, analysis of bone loss requires improved methodologies.

Principal findings: Two ''new'' principles were adopted; any site in infected rats >2 SDs above the mean in control sites defined ''true'' bone loss; any animal with >2 sites in any quadrant with bone loss defined ''real'' disease. Both examiners found bone loss in infected animals greater than controls using these ''new'' principles and DVM ($p<0.05$).

Practical implications: DVM provides a simple, cost-effective and reproducible method for evaluation of bone loss in rodents.