

# Colonization and Persistence of Labeled and “Foreign” Strains of *Aggregatibacter actinomycetemcomitans* Inoculated into the Mouths of Rhesus Monkeys

**Keywords:** *Aggregatibacter actinomycetemcomitans*; Biofilms; Dysbiosis; Experimental animal models; *Macaca mulatta*; Periodontitis; Microbial interactions

## Abstract

*Aggregatibacter actinomycetemcomitans* (Aa) is a pathobiont and part of a consortium of bacteria that can lead to periodontitis in humans. Our aim was to develop a model for oral inoculation of labeled Aa into a suitable host in order to study Aa traits and ecological factors that either enhance or repress its persistence.

Primate species were screened for Aa to select a host for colonization studies. *Macaca mulatta* (Rhesus/Rh) was selected. RhAa strains were isolated, subjected to sequencing and functional analysis for comparison to human strains. “Best” methods for microbial decontamination prior to inoculation were assessed. Three groups were studied; Group 1 (N=5) was inoculated with Aa Spectinomycin resistant (SpecR) Rh strain 4.35, Group 2 (N=5) inoculated with Aa SpecR human strain IDH 781, and Group 3 (N=5) the un-inoculated control. Repeated feeding with pancakes spiked with SpecRAa followed high dose oral inoculation. Cheek, tongue, and plaque samples collected at baseline 1, 2, 3, and 4 weeks after inoculation were plated on agar; 1) selective for Aa, 2) enriched for total counts, and 3) containing 50 µg/ml of Spec. Aa was identified by colonial morphology and DNA analysis.

Rh and human Aa had > 93-98 % genome identity. RhAa attached to tissues better than IDH 781 *in vitro* ( $p < 0.05$ ). SpecR IDH 781 was not recovered from any tissue at any time; whereas, RhSpecR 4.35 was detected in plaque, but never tongue or cheek, in all monkeys at all times ( $> 1 \times 10^5$  colonies/ml;  $p < 0.001$ ).

In conclusion, the primate model provides a useful platform for studying integration of Aa strains into a reduced but established oral habitat. Primate derived SpecRAa was consistently detected in plaque at all collection periods; however, human derived Aa was never detected. The model demonstrated both microbial as well as tissue specificity.

## Abbreviations

Aa: *Aggregatibacter actinomycetemcomitans*; Rh: Rhesus; HACEK: Haemophilus, Actinobacillus, Cardiobacteria, Eikenella, Kingella; BECs: Buccal Epithelial Cells; SHA: Salivary Coated Hydroxyapatite; AAGM: Actinobacillus Actinomycetemcomitans Growth Media; PBS: Phosphate Buffered Saline; SpecR: Spectinomycin resistance; CFU's: Colony Forming Units; NEPRC: New England Primate Research Center



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## Introduction

*Aggregatibacter* (formerly known as *Actinobacillus*) *actinomycetemcomitans* is a Gram-negative bacterium that colonizes the oral cavity of man and primates [1]. This capnophilic bacterium has been highly associated with localized aggressive periodontitis in individuals of African descent [2]. *A. actinomycetemcomitans* is a member of the HACEK (*Haemophilus*, *Actinobacillus*, *Cardiobacterium*, *Eikenella*, *Kingella*) group of bacteria that are also associated with a number of non-oral but systemic infections that include; coronary, kidney, brain, lung and joint diseases in man [3]. All of the HACEK bacteria but particularly Haemophili, Eikenella, and *Aggregatibacter* are found in dental plaque and have been detected on or in oral mucosa and can disseminate via the bloodstream [2]. Therefore, the oral cavity has been portrayed as the portal of entry for these organisms [3,4].

The research focus of our group has been on *A. actinomycetemcomitans* associated host/microbial interactions leading to periodontal bone loss [5]. Along these lines our primary interest is in understanding how *A. actinomycetemcomitans* colonizes teeth, migrates below the gum-line, and avoids immune elimination [6]. Towards these ends our group has developed both *in vitro* assays and *in vivo* animal models to study *A. actinomycetemcomitans* colonization and survival [7,8]. Most recently, we demonstrated that *A. actinomycetemcomitans* leukotoxin, previously thought to be active exclusively in humans and primates, is a prominent virulence factor associated with bone resorption in a rat model of periodontal disease [9].

While we have had significant success in studying colonization in our rat model, the *in vitro* rat lymphocytic response to *A. actinomycetemcomitans* leukotoxin was modest suggesting that the *in vivo* rat model under-represented the bone loss detected [9]. Furthermore bone loss is the end stage of disease [10]. Moreover, there is little doubt that the primates provide a more accurate model of colonization and disease when compared to rodents [11]. For

example, *A. actinomycetemcomitans* adhesins and leukotoxin have been shown to demonstrate similar levels of specificity with respect to human and primate tissue as compared to rodent tissue [12,13]. The main goal of this study is to develop a system that will permit us to implant *A. actinomycetemcomitans* into its “natural” environment such that we can study host and microbial interactions with the newly implanted species overtime. Success in this endeavor will allow us to examine how *A. actinomycetemcomitans* integrates into an existing but reduced oral habitat.

The integration of a “newly” implanted pathobiont into a relatively stable biofilm community can be considered as an important early event in disease development [14,15]. Previously, we have shown that *A. actinomycetemcomitans* can be an early tooth colonizer by demonstrating that it can be detected on an initially sterile hydroxyapatite tooth surrogate within 4-6 hours following placement into the mouths of individuals who harbor *A. actinomycetemcomitans* [16]. This *in vivo* tooth colonization by *A. actinomycetemcomitans* has been shown earlier in several old world primate studies but results from these experiments have not been fully appreciated and as a result *A. actinomycetemcomitans* is still considered by many to be a poor tooth colonizer [17-19]. Further documentation of early colonization by *A. actinomycetemcomitans* in humans is problematic because; 1) *A. actinomycetemcomitans* is not typically detected as a prominent member of the indigenous oral flora in periodontally healthy individuals, 2) the percentage of individuals colonized by *A. actinomycetemcomitans* is low, and 3) very little flexibility can be tolerated with respect to either microbial or environmental manipulation when humans are involved [3].

It is our contention that we can overcome these impediments by studying *A. actinomycetemcomitans* colonization in a mammalian environment similar to humans that is also hospitable for *A. actinomycetemcomitans* growth and survival. Such an environment is routinely found in primates [20]. Therefore, a primate model is appealing for the following reasons; 1) the oral environment supports the growth of *A. actinomycetemcomitans*, 2) microbial genes of implanted *A. actinomycetemcomitans* strains can be manipulated, 3) interaction of the resident flora can be studied and manipulated, and 4) host interactions can be studied and altered by factors such as smoking, antibiotics, hormones, foods etc.

Thus a model permitting the integration of a “foreign”, labeled, and newly implanted bacterium into a complex biofilm community could help address several questions. For example, tracking an implanted and labeled reference strain of *A. actinomycetemcomitans* can help describe both disruption and/or integration into an existing complex, compact microbial community [21,22]. This is a compelling line of investigation since all mucosal infections occur in a competitive environment and disruption or dysbiosis of that community forms the basis of the first step in the initiation of bacterially induced mucosal infections [3]. In this manuscript we describe the procedures and results derived from our studies that were designed to determine the best method and most appropriated *A. actinomycetemcomitans* strain capable of integrating into a microbial community that has the ability to support *A. actinomycetemcomitans* colonization, growth and survival, and/or elimination.

## Methods and Materials

### Survey of Primate oral flora and selection of *A. actinomycetemcomitans* strains for use in the primate model

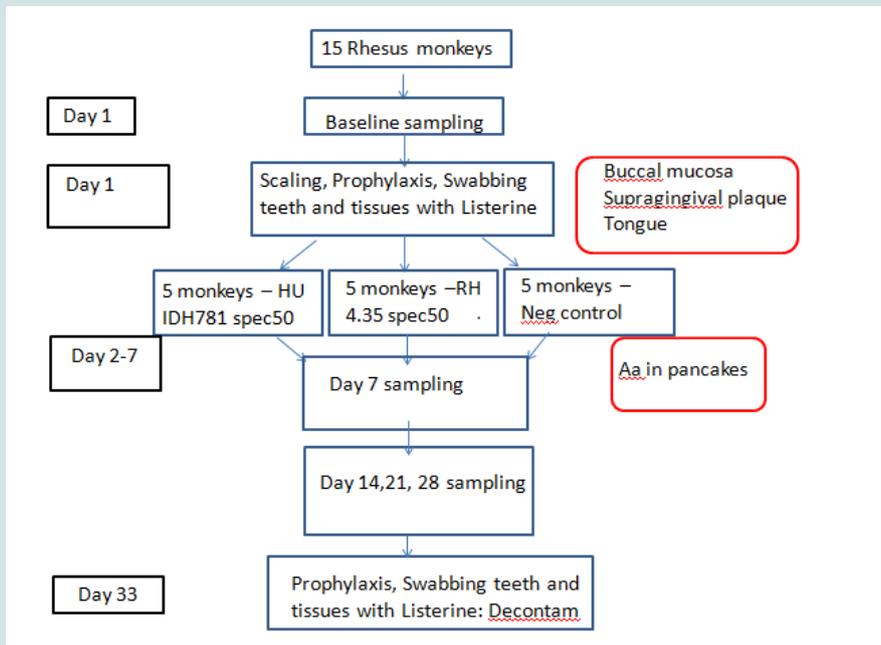
The Institutional Animal Care and Use Committees (IACUC) at Rutgers, the New England Primate Research Center (NEPRC) and the Southwest Primate Center approved all work with primates. In all experiments, prior to anesthetizing primates, vital signs and weights were obtained. Monkeys were monitored on a daily basis as per IACUC recommendations. In cases where “foreign” strains of *A. actinomycetemcomitans* were implanted; procedures were performed at the conclusion of experiments to assure that these strains were no longer detectable (see Figure 1).

Initially we screened over 80 *Macaca mulatta* (Rhesus; Rh) monkeys, 40 marmosets, and an assortment of other primates including; Chimpanzees (n=8), *Cynomolgus* (n=4), Baboons (n=4), and Tamarins (n=4) from three primate centers to determine the percentage of primates who harbored *A. actinomycetemcomitans*. *A. actinomycetemcomitans* was isolated from both buccal tissue and plaque obtained from each of the 140 primates screened. These samples were then grown on AAGMBV agar and then specific colonies with the characteristic colonial morphology were picked from plates and tested using biochemical and DNA methods for identification. We then subcultured several *A. actinomycetemcomitans* stains and extracted DNA to do analysis of selected virulence traits for our initial comparative genomic assessment [7]. More than 80% of the Rh monkeys sampled had *A. actinomycetemcomitans* in their dental plaque [20]. Our goal was to introduce a labeled stain of *A. actinomycetemcomitans* into a complex competitive environment that was capable of supporting its growth and survival.

Of the primates screened, Rh monkeys appeared to provide the best environment (see Table 1) for our experimental goals [20]. We collected several dozen *A. actinomycetemcomitans* strains with a range of serotypes from the Rh primates at the NEPRC (Table 1). We compared the attachment characteristics of several of these primate strains to human *A. actinomycetemcomitans* strain IDH 781, considered an outstanding colonizing bacterium *in vitro* and in our rat studies [9,13]. Initially we compared binding of various *A. actinomycetemcomitans* primate and human strains to buccal epithelial cells (BECs) and salivary-coated hydroxyapatite (SHA). Several primate strains were sequenced and compared both for their complete genomes and also for a variety of virulence genes of interest [3,4]. For purposes of this study we focused our comparison of *A. actinomycetemcomitans* strains on attachment to soft and hard tissue since our goal was colonization.

### Buccal Epithelial Cell (BEC) binding assay

This assay has been described previously [7]. Briefly, we collected buccal cells from Rh monkeys by scraping the side of the cheek gently but firmly to capture enough cells to obtain  $5 \times 10^4$  BECs per ml. We suspended these cells in phosphate buffered saline (PBS) and added *A. actinomycetemcomitans* cells (either Rh or human strains) at a concentration of  $1 \times 10^8$  ml. Prior to mixing, the *A. actinomycetemcomitans* suspension was subjected to sonication for 15 seconds at a low power output to break up clumps of cells.



**Figure 1: Flow Diagram illustrating the overall inoculation, colonization and persistence protocol.** Bacteria were collected from teeth at baseline. Animals were divided into three groups. After debridement and decontamination, inoculation was performed followed by pancake feeding on days 2 to 5. Samples were collected on weeks 1 to 3 using a buccal brush passed over one quadrant. A brush was passed over the cheeks and tongue. Brush samples were stabbed into an agar containing tube, shipped to Rutgers and samples were then plated on various media. On week 4 (28 days) samples were collected from all surfaces by scaler and vigorously with the buccal brush for plating. On day 33 decontamination was performed and on day 60 samples were collected by buccal brush and shipped for analysis of residual labeled Aa. One of the controls was dropped due to medical complications unrelated to the study. Thus 4 controls completed the study.

Table 1: Primates surveyed for *A. actinomycetemcomitans*

Monkey group	Number surveyed	Sex (M/F)	#Aa positive	Serotype
Rhesus	80	36/44	75	a=18, b=10, c=7 d=9, e=5, f=26
Chimpanzee	8	2/6	4	c=1, b=2, d=2
Cynomolgus	4	4/0	0	
Baboon	4	1/3	0	
Tamarin	4	2/2	0	
Marmoset	40	22/18	2	f=1, d=1

The mixture was allowed to incubate for 30 minutes to allow for binding of *A. actinomycetemcomitans* to BECs. The mixture of *A. actinomycetemcomitans* cells and BECs was then placed over a tube containing a 5% Ficoll 400 gradient, which was subjected to low speed centrifugation (50-x g for 5 min) to separate the heavier epithelial cells from the bacteria. The supernatant was removed and the pellet containing the BECs was plated on a medium containing AAGM agar with Bacitracin and Vancomycin (AAGMBV) to determine the number of *A. actinomycetemcomitans* bound per BEC [16]. Primate strains RHAA, Rh 1.4, Rh 2.3, Rh 3.8, Rh 4.35 and human strain IDH 781 were used in binding studies. Controls consisted of *A. actinomycetemcomitans* alone, BECs alone, and BECs mixed with non-adherent lab strains of *A. actinomycetemcomitans*.

### Binding to hydroxyapatite (HA)

This assay has also been described previously [7,23]. Briefly, a suspension of *A. actinomycetemcomitans* (both human and Rh derived) was obtained as described above to achieve an optical density  $A_{560} = 0.9$  (equivalent to  $1 \times 10^8$  cells/ml) [7]. Whole unstimulated saliva was collected from healthy subjects (an IRB from Rutgers University was used for consent) and clarified by centrifugation at 10,000-x g to obtain 1 ml of saliva for addition to 50 mg of hydroxyapatite beads (BDH Chemicals Ltd. Poole, UK). After 1 hour, beads were washed three times with KCl (0.05 mM KCl, 1 mM potassium phosphate, pH 6.0, 1 mM calcium chloride, 0.1mM magnesium chloride) (Sigma). For attachment studies, stocks of human IDH 781 and Rh 4.35 *A. actinomycetemcomitans* were grown for 24 hours in AAGM broth.

A 100µl sample of the suspension was added to 50 mg of saliva coated beads and the mixture of beads and bacteria were placed on a rotating device (RotoTorque from Cole Palmer Instruments, Chicago IL) for 30 minutes. Beads were allowed to settle for 2 minutes and the supernatant containing the unbound bacteria were removed and plated on AAGM agar. The pellet containing the cells bound to SHA was then washed three times in phosphate buffered saline (PBS). After the final wash, cells were sonicated, centrifuged at low speed and serially diluted, resuspended in 1 ml of PBS, and plated on AAGM agar to determine *A. actinomycetemcomitans* cells bound to SHA. The ratio of bound to unbound cells was calculated as described [7,23]. Binding of primate strain Rh 4.35 was compared to human strain IDH 781.

### Competitive binding to BECs

Attachment of *A. actinomycetemcomitans* to BECs is dependent on specific adhesin/receptor interactions that; 1) require affinity, and 2) reach a concentration dependent binding plateau. Reversibility of Rh 3.8 *A. actinomycetemcomitans* binding to BECs was challenged by other strains of *A. actinomycetemcomitans* derived from Rh monkeys (Rh 4.35) and human *A. actinomycetemcomitans* (IDH 781). The experiment was designed to determine if one strain of monkey Rh (4.35 SpecR) could replace the already bound Rh *A. actinomycetemcomitans* (Rh 3.8) in vitro in comparison to a human SpecR *A. actinomycetemcomitans* (IDH 781). We thought these results might serve as a possible prelude to what might happen in the mouths of monkeys. Specifically, after an initial BEC binding experiment where Rh BECs were treated with unlabeled Rh 3.8 *A. actinomycetemcomitans* for a 30-minute incubation period, the reaction mixture (Rh *A. actinomycetemcomitans* plus Rh BECs) was divided into three equal aliquots of 500 µl. One aliquot was used as the control and reacted with 500 µl of the same Rh 3.8 strain, one was reacted with 500 µl of SpecR labeled Rh *A. actinomycetemcomitans* (see labeling method below) derived from a different monkey strain (Rh 4.35) and the third was reacted with 500 µl of IDH 781 SpecR *A. actinomycetemcomitans*. All three mixtures were then incubated for another 30 minutes. After this second incubation period each reaction mixture was placed over a Ficoll gradient in preparation for assessment of SpecR *A. actinomycetemcomitans* binding per BEC. The goal was to determine the ability of added and labeled *A. actinomycetemcomitans* (human or primate) to displace the original primate *A. actinomycetemcomitans* (Rh 3.8) that was already bound to primate BECs [24].

### Labeling of primate and human *A. actinomycetemcomitans* strains

IDH 781 and Rh 4.35 were labeled by creating Spectinomycin resistance (SpecR). Spectinomycin was recommended as the antibiotic of choice for labeling by the veterinarians via the IACUC because this antibiotic was not needed for treatment or other purposes. Labeling (resistance) was developed by passaging the strains on successive plates of agar containing increasing amounts of Spectinomycin until they demonstrated a level of resistance to 50 µg/ml of Spectinomycin. After successful labeling was attained, binding to BECs and SHA was repeated to determine whether the labeling had any effect on these important phenotypes.

### Selection of an oral decontamination method

In preliminary experiments we were able to assess three different cleaning methods to reduce the biofilm that existed on the teeth prior to inoculation of the test *A. actinomycetemcomitans* strains using a subset of three primates per experiment. The first method consisted of scaling all teeth using a typical manual scaling procedure followed by inoculation of the labeled *A. actinomycetemcomitans* test strain. The second method included scaling, followed by rubber cup prophylaxis prior to inoculation. The third method consisted of scaling, rubber cup prophylaxis, followed by topical application of a local antiseptic (Listerine antiseptic) followed by rinsing with sterile water to remove the residual antiseptic prior to inoculation.

### Selection of inoculation and feeding method

After cleaning and local inoculation of *A. actinomycetemcomitans* we fed the animals repeatedly to continue exposure to the inocula for the next 4 days. Both primate and human *A. actinomycetemcomitans* strains were first tested *in vitro* by adding *A. actinomycetemcomitans* to various foods to determine the best food substance to use for repeated inoculation so as to avoid the need to anesthetize the animals on re-inoculation. The foods assessed were banana, marshmallow, graham crackers, and pancakes. To test survival, one ml of a dose of  $1 \times 10^8$  cells per ml were added to each food source and the *A. actinomycetemcomitans* was allowed to sit in the food source at room temperature for 0 minutes, 30 minutes and 120 minutes. Foods were then mashed and diluted in AAGM broth and tested for growth on AAGMBV agar cultured at 37 °C for three days and then *A. actinomycetemcomitans* colony forming units per ml (CFU's/ml) were counted.

### Selection of an inoculation method

To determine the best method of inoculation we tested feeding alone vs. direct inoculation and feeding.

### Experimental Design

The purpose of the initial full experimental protocol was to determine whether a human *A. actinomycetemcomitans* strain or a primate *A. actinomycetemcomitans* strain would colonize the oral cavity of primates and survive for a period of 4 weeks. (Figure 1; Flow chart). Three groups of monkeys were used as follows; Group 1 had 5 Rh monkeys who were inoculated with SpecR labeled human strain IDH 781, Group 2 had 5 Rh monkeys who were inoculated with SpecR labeled primate strain Rh 4.35 serotype b, and Group 3 consisted of 5 Rh monkeys that were un-inoculated. Monkeys were anesthetized with 10-20 mg/kg of ketamine and medetomidine intramuscularly, brought to the laboratory suite, and then each monkey was sampled for total bacteria and recovery of indigenous *A. actinomycetemcomitans*. Two independent samples were taken from teeth, tongue and cheek; one for DNA analysis and one for cultural analysis of *A. actinomycetemcomitans* indigenous to the oral cavity of each of the primates participating in the study. Following this teeth were cleaned with scalers and then polished with a rubber cup and pumice. After polishing, teeth were washed with water, followed by application of fully concentrated Listerine to all teeth and soft tissue surfaces by means of a soaked cotton applicator. Sterile water was then applied to all surfaces to remove the Listerine. Shortly thereafter

a confluent suspension of the *A. actinomycetemcomitans* strains (IDH 781SpecR or Rh 4.35 SpecR) was applied to all teeth and tissue surfaces until the full 1 ml of a concentration of  $1 \times 10^8$  cells/ml was inoculated. Prior to inoculation the dense suspension was subjected to agitation to break up clumps. Teeth and tissue were doused by repeated topical application of a total of 1 ml of the fully dispersed thick suspension of bacteria applied to teeth and soft tissues. To subject animals to repeated inoculation, pancakes (Aunt Jemima) were then spiked with *A. actinomycetemcomitans* on one side and with pancake syrup on the opposite side for feeding of primates on days 2-5. Pancake fed to control animals had no *A. actinomycetemcomitans*.

### Sampling and identification of *A. actinomycetemcomitans*

Animals were anesthetized and sampled as described at weeks 1, 2, and 3 using a buccal brush collection method. In this method the brush was passed over a limited area of tissue or tooth several times to collect sufficient material. Individual brushes were used to obtain samples from tooth, cheek, and tongue. After collection, brushes were stabbed into AAGMBV agar for recovery of *A. actinomycetemcomitans*. Samples were shipped overnight from the NEPRC to Rutgers. Samples recovered from stabs were plated on AAGMBV, Spec and enriched trypticase soy agar (ETSA) for analysis. *A. actinomycetemcomitans* was identified by; colonial morphology, biochemical testing, and DNA analysis [25]. Plaque samples were removed from teeth by dental scalers in the week 4-collection period (day 28), while tissue was sampled by buccal brush. This sampling was vigorous and thorough. On day 33 full prophylaxis and decontamination was performed to eliminate the inoculated SpecR *A. actinomycetemcomitans* (Figure 1). A final sample was taken on day 60 to insure that the inoculated *A. actinomycetemcomitans* strains were eliminated.

### Microbiological analysis

Two samples were taken from each of the oral surfaces; one for DNA analysis, and one for cultural analysis of CFU's/ml. Samples for DNA analysis were refrigerated and then frozen for future study. Each sample taken for cultural analysis was place in 1 ml of PBS and then plated on the following agar; ETSA with 5% blood, AAGMBV agar for enumeration of total *A. actinomycetemcomitans* and AAGM with 50 µg/ ml Spec for enumeration of SpecR strains of *A. actinomycetemcomitans* [7,20,26].

### Statistical analysis

Selection of the best method for preparation of the oral cavity, for *A. actinomycetemcomitans* inoculation, and feeding was descriptive in nature and based on a small subset of monkeys and thus determined by inspection of data by members of the research team. Survey of the presence of *A. actinomycetemcomitans* in the mouths of the various primates analyzed from the three primate centers was presented in tabular form as descriptive data with no statistical analysis although it was clear without statistical analysis that Rh primates had the highest carriage of *A. actinomycetemcomitans*.

Data derived from *in vitro* binding assays (BEC) were analyzed by ANOVA using a Fisher exact test to determine the level of binding and using a Chi-square to determine best binding /when comparing the human and monkey strains to attain a significance level of 0.05.

SHA binding was assessed by means of a two-way students-t-test setting the significance level at 0.05.

Data derived from the full experimental *in vivo* protocol designed to assess the presence or absence of SpecR *A. actinomycetemcomitans* in the primate samples was analyzed by ANOVA using the Kruskal Wallis analysis of variance by ranks to compare the three groups and to determine whether human or primate *A. actinomycetemcomitans* was better in colonizing *in vivo*. The un-inoculated group acted as the control in the analysis with the significance value set at  $p < 0.05$ .

## Results

### Survey of primates for possession of *A. actinomycetemcomitans*

In Table 1 we present the data from our survey of over 80 Rhesus monkeys, 40 marmosets, 8 chimpanzees and 4 cynomolgus, 4 Baboons, and 4 Tamarins. While 57% of the primates sampled had *A. actinomycetemcomitans*, over 90 % of the Rh monkeys surveyed had *A. actinomycetemcomitans*. Moreover, all major serotypes of *A. actinomycetemcomitans* in the Rh primates were detected and 80% of the Rh primates screened had only one serotype of *A. actinomycetemcomitans*. Fifty percent of the chimpanzees had *A. actinomycetemcomitans* and only 5% of the marmosets had *A. actinomycetemcomitans*. We could not recover *A. actinomycetemcomitans* strains from the other primates evaluated (Table 1). Serotype distribution was seen as follows; “a”, and “f” were dominant but “b”, “d” and “c” were numerous (Table 1). It should be pointed out that none of the isolated strains showed the JP2 promoter region deletion.

### Selection of human and monkey *A. actinomycetemcomitans* strains for study

Based on our survey and pragmatic considerations we decided to use Rh monkeys from the NEPRC for our study and initially chose a serotype b strain of *A. actinomycetemcomitans* (Rh 4.35) for inoculation. We performed an overall genome sequence analysis of “a”, “b”, “c” and “d” serotypes from *A. actinomycetemcomitans* strains isolated from Rh monkeys from the NEPRC primate center [27]. We also chose IDH 781, a human “d” serotype *A. actinomycetemcomitans* strain for our comparison in the inoculation studies because of its success in our *in vitro* binding assays and in our *in vivo* rat studies [9].

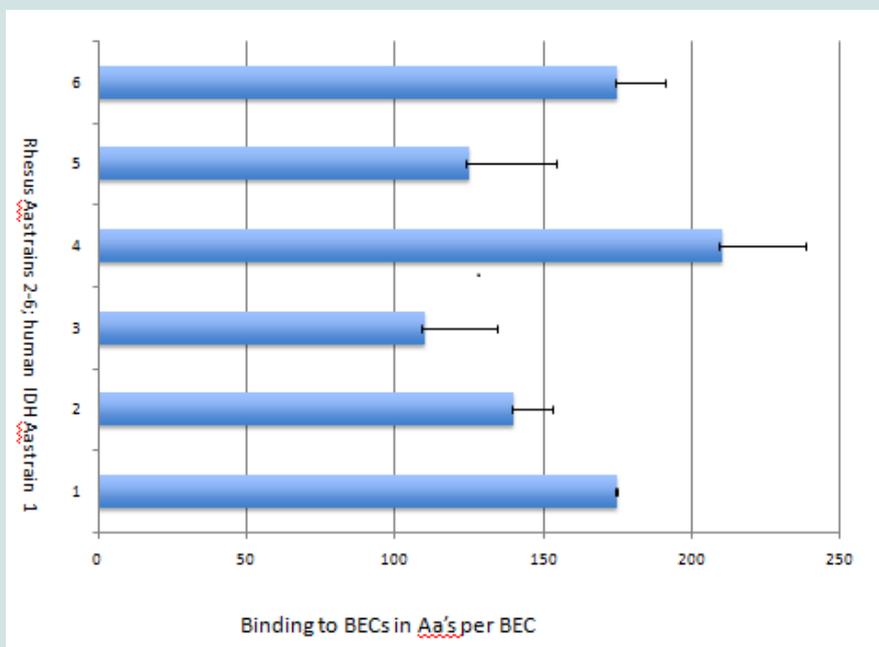
### DNA comparison and SHA and BEC binding comparisons

After strain selection we performed DNA analysis of the genomes of eight primate strains as well as DNA and amino acid comparison of the specific virulence traits of these strains to those found in humans [27]. As such we compared human and primate strains for adhesin and attachment genes *aae*, *apiA*, *flp* and *tadA*, other virulence genes were also compared as well as an assortment of housekeeping genes. Initial analysis indicated that strains assessed had a minimum of 98% nucleic acid identity and from 93-99% sequence identity in all virulence traits analyzed suggesting that the microbes were comparable (data not shown). Since we felt that attachment characteristics were clinically relevant for our *in vivo* colonization experiments we compared binding properties in several primate strains and compared these properties to IDH 781 for both soft and hard tissue binding. In BEC binding no statistical differences were seen when most Rh strains were compared although strains Rh 4.35

bound better than RHAA and Rh 2.3 ( $p < 0.05$ ) while comparisons to other Rh strains were not significant (Figure 2). Further in a competitive soft tissue-binding assay it was shown that Rh 4.35 monkey derived *A. actinomycetemcomitans* displaced Rh 3.8 monkey *A. actinomycetemcomitans* bound to monkey BECs by 69.8% (Table 2). These results were significantly superior to the displacement of bound Rh 3.8 *A. actinomycetemcomitans* by human IDH 781 *A. actinomycetemcomitans* which was at a level of 50.2% ( $p > 0.05$ ; Table 2). Further in SHA binding comparisons bound to unbound ratios were significantly higher in primate strain Rh 4.35 ( $3.88 \pm 0.45$ ) as compared to human strain IDH 781 ( $1.98 \pm 0.25$ ) (Figure 3,  $p < 0.05$ ). We also compared binding of the SpecR strains to their parent unlabeled strains for both hard and soft tissue binding. No significant differences were seen when labeled strains were compared to unlabeled strains (data not shown).

**Choice of food for repeated inoculation after cleaning**

As seen in Table 3 comparison of survival of *A. actinomycetemcomitans* when placed on banana, marshmallow, graham crackers and pancakes showed that *A. actinomycetemcomitans* survival overtime was superior on pancakes and thus 74% of *A. actinomycetemcomitans* were still viable after 2 hours. Prior to these *in vitro* experiments we gave monkeys each of the food substances as recommended by the veterinarians because the animals are known to be very particular about the form, shape, and smell of the foods they eat. To make the *A. actinomycetemcomitans* spiked pancakes more appealing we placed syrup on the side of the pancake free of the inoculum. This approach was well accepted by all the monkeys in the study.



**Figure 2: Binding of various *A. actinomycetemcomitans* strains to Rh buccal cells *in vitro*.** A variety of strains of *A. actinomycetemcomitans* were reacted with buccal cells obtained from Rh monkeys to compare epithelial cell binding. Results indicated that #6 (Rh strains 3.8) and #4 (Rh strain 4.35) were most similar to #1 (human strain IDH 781) although significant differences in binding was obvious only when strains #4 (Rh 4.35) were compared to strains #3 (RhAA) and #5 (Rh 2.3). None of the other comparisons demonstrated statistical differences.

Table 2: Replacement of Rhesus *A. actinomycetemcomitans* bound to Buccal Epithelial Cells (BECs)

	Aa Counts	% Replacement
Initial Attachment of Monkey Aa:BEC	169.2 ± 2.6	
Replacement by IDH781Spec <sup>R</sup>	84.6 ± 3.5	50.2%
Initial Attachment of Monkey Aa:BEC	154.2 ± 3.6	
Replacement by Rh Spec <sup>R</sup>	107.7 ± 1.1	69.8%*

\* Replacement by RhSpec<sup>R</sup> is significantly higher than IDH781Spec<sup>R</sup>;  $p < 0.05$

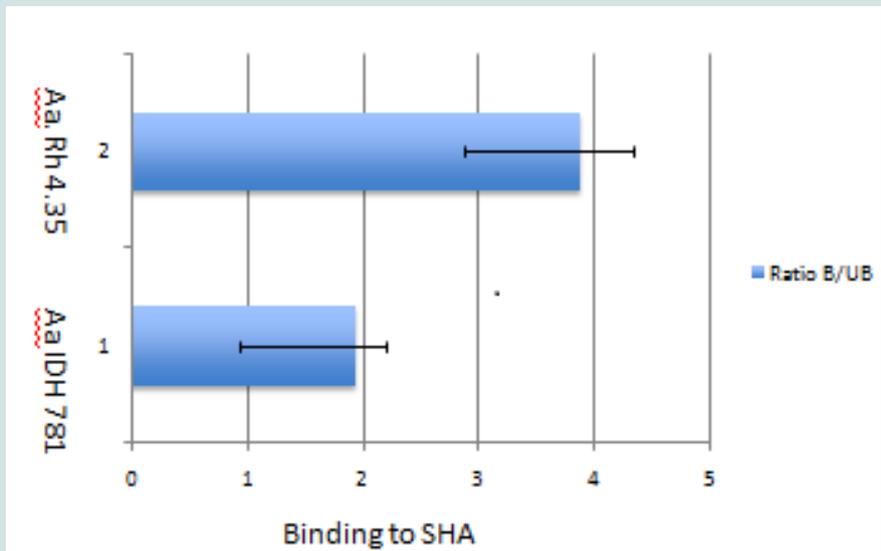


Figure 3: Binding of *A. actinomycetemcomitans* strains to salivary-coated hydroxyapatite. Strains Rh 4.35 was compared to IDH 781. When the ratio of Aa cells bound to SHA to unbound cells in #2 (Rh 4.35) the ratio was  $3.88 \pm 0.45$  while the ratio for IDH 781 was  $1.94 \pm 0.25$ ;  $p < 0.05$ .

Table 3: Survival of *A. actinomycetemcomitans* in various foods over time

Time (min)	Banana	Marshmallow	Graham Cracker	Pancake
0	100.0%	100.0%	100.0%	100.0%
30	94.4%	89.8%	0.06%	100.0%
120	20.3%	57.6%	0.0%	74.3%

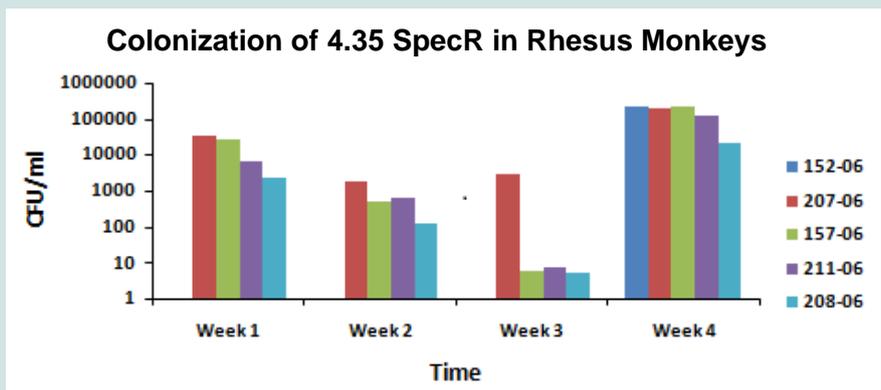


Figure 4: Graphic illustration of colonization and persistence of labeled *A. actinomycetemcomitans* in plaque collected over 4 weeks after inoculation. All 5 Rh monkeys showed a reasonable level of binding of Rh 4.35 SpecR monkey derived *A. actinomycetemcomitans* to tooth surfaces. Binding to tongue or to cheek was never detected. No human strain of *A. actinomycetemcomitans* was ever recovered from any surface any time point. Frequency of binding to teeth was highly significant when the inoculated monkey *A. actinomycetemcomitans* strain was compared to the human strain. Using a Fisher's exact test the P value for comparison was 0.0002.

**Preparation for inoculation: debridement, feeding and inoculation methods**

In pilot studies we compared three forms of debridement and the method that combined manual scaling, rubber cup prophylaxis with prophylaxis paste, and topical application of Listerine, and immediate rinsing with water, provided the best preparatory

method prior to inoculation of the “foreign” antibiotic labeled *A. actinomycetemcomitans* (data not shown).

In the initial pilot studies we used human strain IDH 781 SpecR as the inoculum and compared implantation and feeding methods that consisted of topical inoculation alone or in combination with feeding procedures that involved *A. actinomycetemcomitans* spiked pancakes.

In these pilot studies we assessed survival over a two-week period following debridement. Our data indicated that only the inoculation and repeated feeding protocol was successful but colonization of the teeth was very sparse and occurred in only one of the three monkeys (data not shown).

### **Recovery of labeled primate or human *A. actinomycetemcomitans* from oral cavity of primates using the full four-week experimental protocol**

As seen in Fig 4 monkey *A. actinomycetemcomitans* (Strain Rh 4.35) was recovered at all time points from the 4 of the 5 monkeys and in all 5 monkeys at week 4. Importantly, at no time was there any evidence that labeled *A. actinomycetemcomitans* derived from humans (IDH 781 SpecR) could be recovered from any collection site. It should be noted that collection at weeks 1 to 3 were done by means of a collection brush that was swiped along the teeth from one quadrant in one case, along the tongue in the second case, and along the cheek in the third collection. Collection at week 4 was done with a scaler and collection was not limited to any tooth surface, hence full mouth plaque collection should have increased the efficiency of the recovery. Nevertheless, despite modest collection procedures performed on weeks 1 to 3, labeled *A. actinomycetemcomitans* derived from primates was detected in plaque in 4 of the 5 monkeys during that time period. The one Rh monkey where there was a failure to detect labeled *A. actinomycetemcomitans* did show *A. actinomycetemcomitans* at the final 4-week collection time. The implanted SpecR *A. actinomycetemcomitans* colonization level represented anywhere from 1 to 10% of the total *A. actinomycetemcomitans* flora recovered (data not shown). However, no labeled *A. actinomycetemcomitans* was collected from primate tongue or cheek at any time-point.

### **Discussion**

We had previously developed a system designed to study *A. actinomycetemcomitans* in a Specific Pathogen Free rat model where we had created antibiotic labeled *A. actinomycetemcomitans* strains that were inoculated into the mouths of rats with a reduced oral flora [7]. Over the years we have been able to identify factors required for *A. actinomycetemcomitans* colonization and persistence in this model [28]. These studies have shown the importance of adherence factors and leukotoxin even in an animal system where *A. actinomycetemcomitans* is not routinely found [9]. These studies have also indicated that different rat strains show differing sensitivity to the same strain of *A. actinomycetemcomitans* and that differing strains of *A. actinomycetemcomitans* produce different levels of disease in the same rat strain [9,28]. Results from these rat studies are very similar to results seen in studies of *Porphyromonas gingivalis* induced periodontal disease in mice [29,30]. These rodent studies parallel human observational studies showing that not all humans infected with *A. actinomycetemcomitans* succumb to disease [26,31]. However, since *A. actinomycetemcomitans* is not typically found in the mouths of Specific Pathogen Free rats it is unlikely that these rodent studies will uncover information related to colonization resistance, the impact other members of the indigenous dental biofilm flora have on *A. actinomycetemcomitans* growth and survival, or how leukotoxin protects *A. actinomycetemcomitans* from PMNs, macrophages and lymphocytes [4,32].

Recently Hogland Aberg et al. have shown that *A. actinomycetemcomitans* leukotoxicity substantially enhances the Odds Ratio for onset and progression of LAP in adolescents colonized with *A. actinomycetemcomitans* [33]. The relationship between leukotoxin and onset and progression of LAP was originally shown by Haubek et al. and this positive correlation between *A. actinomycetemcomitans* leukotoxic strains and aggressive periodontal disease has been well established over the last several years [31,34]. It is unlikely that the response to *A. actinomycetemcomitans* leukotoxin in rats can be compared to lymphocytes derived from primates and/or humans [4]. Since the specificity of leukotoxic activity is similar in primates and humans, the primate model is ideal for studying *A. actinomycetemcomitans* and its relationship to disease. Further, the primate model can help address the question as to how moderate to low leukotoxin producing strains of *A. actinomycetemcomitans*, are related to aggressive periodontal disease in subjects who do not harbor the high leukotoxin producing strains of *A. actinomycetemcomitans* [26]. In this case it is likely that a key element in disease progression relates to other members of the flora that either support or interfere with *A. actinomycetemcomitans* colonization and persistence [26]. This is also best accomplished in an environment that closely resembles that seen in the human mouth. In contrast to what we see in Specific Pathogen Free rats, *A. actinomycetemcomitans* is a natural inhabitant of the oral flora of the *Macaca mulatta* (Rhesus macaques).

Our assumptions related to *A. actinomycetemcomitans* in the mouths of primates is supported by early work by Taichman and colleagues, Beighton et al. and Kilian and Rolla and a number of studies by Ebersole and associates all indicating that *A. actinomycetemcomitans* is routinely present in the mouths of old world primates [11,12,17,18]. As such, implanting a labeled strain of *A. actinomycetemcomitans* into the mouth of an animal that supports its growth, survival and response to key virulence factors (i.e. leukotoxin) overtime should allow us to study; 1) factors that support *A. actinomycetemcomitans* growth and colonization, and perhaps more importantly 2) factors that restrict *A. actinomycetemcomitans* growth and colonization. Thus the first goal, in the first of several studies, was to establish a reliable model system that would allow us to track *A. actinomycetemcomitans* overtime in an oral cavity with anatomic and environmental similarities to that found in man [12].

To accomplish this goal we had to develop the optimal conditions for delivery of a “foreign and labeled” *A. actinomycetemcomitans* strain into the oral cavity of a group of Rh monkeys that were individually housed. It was our contention that selection of the appropriate Rh strain for comparison to our well studied human strain IDH 781 could provide us with information concerning specific *A. actinomycetemcomitans* virulence traits that support or inhibit its growth and survival in a competitive environment [21,35].

Our first effort was to determine general genomic and functional similarities or differences in key adherence properties in human and primate *A. actinomycetemcomitans* strains chosen as potential candidate strains for implantation into the mouths Rh monkeys [27]. Rh strain 4.35 was selected by virtue of its attachment characteristics. After choosing this strain we sought to conduct studies aimed at demonstrating reproducible ways of removing or significantly reducing members of the resident flora from tooth and tissue surfaces

that might impede colonization of our implanted strain. By reducing the indigenous flora we envisioned that we would provide our implanted strain with a survival advantage. These methods proved to be less straightforward than anticipated. Only methods that included intense scaling and prophylaxis followed by application of topical antiseptics allowed for sufficient microbial reduction to permit colonization of the newly implanted *A. actinomycetemcomitans* strains. These difficulties were not totally unexpected since the clinical literature is replete with indications regarding the difficulty clinicians have in removal and elimination of *A. actinomycetemcomitans* from the oral cavity of those infected with *A. actinomycetemcomitans* [1,36-38]. Nevertheless, the intensity of the recolonization of native *A. actinomycetemcomitans* was surprising in light of the massive inoculation procedure we used to implant the “foreign” *A. actinomycetemcomitans*.

Most interesting is the fact that the human strains of *A. actinomycetemcomitans* inoculated into the mouths of the monkeys were not seen at any time over the 4 week period while all 5 monkeys inoculated with the monkey strain of *A. actinomycetemcomitans* were colonized. These colonies persisted at reasonable levels throughout the 4-week period. It should be mentioned that feeding alone was not sufficient but topical inoculation of a very high dose of *A. actinomycetemcomitans* immediately after scaling, prophylaxis and antiseptic application followed by repeated pancake feeding for 5 days did suffice. Also it should be noted that *A. actinomycetemcomitans* was consistently recovered from tooth surfaces but never from the tongue or cheek surfaces. It is possible that the primate strain chosen possessed superior attachment characteristics when compared to the human strain chosen, although this same human strain had outstanding binding properties *in vitro* and was successful in its ability to colonize rats in previous experiments [28]. These results suggest a high level of species and tissue or ecological specificity. Exactly why the human and monkey strains of *A. actinomycetemcomitans* performed so differently *in vivo* in the implantation experiments is still to be determined. It is also interesting to note that the implanted and successful colonizing strain (Rh 4.35) only formed a low percentage of the total *A. actinomycetemcomitans* found in monkey plaque. This is especially interesting because of the high dose and repeated inoculation protocol followed in these experiments.

These results suggest that precise discriminatory events take place in the *in vivo* environment that protects mammals from invasion of alien species (in this case a human strain of *A. actinomycetemcomitans*) into a biofilm community. This discrimination occurs even when the implanted species shows a high level of similarity to the native strains and even though the indigenous microbial community has been severely compromised by physical and chemical means [4,39].

In conclusion, we feel that we have successfully developed an animal model that has many similarities to the human condition and thus can be used for implantation and study of factors that can influence *A. actinomycetemcomitans* colonization. The data from this study indicates that intensive physical and chemical cleaning needs to be performed prior to implantation. The data also suggests that implantation is best accomplished by high dose local inoculation of freshly grown strains followed by a feeding protocol that continues the inoculation regimen. Further, this study clearly demonstrates that a monkey strain as opposed to a human strain has superior success

in colonization and persistence in the oral cavity of a primate host. With these results in hand we feel that we are positioned to study the deletion of specific *A. actinomycetemcomitans* virulence genes as well as the effect of commensal bacteria on colonization and survival in the context of a fully developed but reduced oral flora.

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