ORIGINAL ARTICLE

# Detection of *T. forsythia* and other important bacteria in crestal and subcrestal implants with ligature-induced peri-implant infection in dogs

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## Abstract

**Background:** The study was designed to compare peri-implant microbial colonization of inflamed implants placed at different levels in dogs.

**Methods:** Two screwed-in (SI) and two tapped-in (TI) conical connection implants were placed on each side of mandibles in six dogs respectively. Four experimental groups were constituted according to implant types and placement depth in one side: SI crestally (SIC), SI 1.5 mm subcrestally (SIS), TI crestally (TIC), and TI 1.5 mm subcrestally (TIS). Plaque accumulation of implants was promoted by cotton ligatures at either side randomly selected in each dog four weeks after abutment connection. Peri-implant sulcular fluid (PISF) samples were collected at 4 weeks, 10 weeks, and 16 weeks after abutment connection. Common periodontal pathogens in PISF were analyzed by PCR and realtime-PCR to investigate the influence of placement depth on microbial accumulation. The microbial results were further correlated with clinical parameters.

**Results:** At ligatured sides, detection rates of *T. forsythia* and *P. gingivalis* increased significantly in four groups. *T. forsythia* levels increased significantly from baseline in four groups at ligatured sides at 16 weeks ($p < 0.05$). TIS group harbored significantly more *T. forsythia* than TIC at ligatured sides at 16 weeks ($p < 0.05$). At ligatured sides, probing depth was correlated to *T. forsythia* level in four groups as well as in total.

**Conclusions:** Subcrestal placement could increase the peri-implant *T. forsythia* level at the early stage of peri-implantitis. The *T. forsythia* level in the peri-implant sulcus is associated with probing depth.
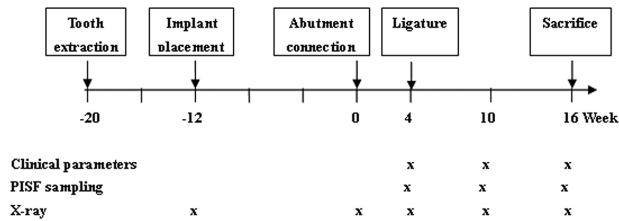
### KEYWORDS

animal experimentation, dental implant-abutment design, peri-implantitis, polymerase chain reaction, *Tannerella forsythia*

Peri-implantitis is the peri-implant inflammation involving soft and hard tissue. A systematic literature review has shown the 5-year incidence of peri-implantitis varies from 12% to approximately 43% depending on the diagnostic criteria.[1,2] According to the consensus report from the Seventh European Symposium on Periodontology, the occurrence and development of peri-implantitis is closely related to the peri-implant microbes,[3] and rapid colonization of bacteria has been observed at the peri-implant sulcus after the placement of titanium implants.[4]

The microgap at the implant-abutment-interface (IAI) is a key factor influencing the peri-implant microbes, and the placement depth decides the relative location of IAI microgap with hard and soft tissue. Histomorphologic results from

**FIGURE 1** Flowchart of the animal study



**FIGURE 2** Surgical image of implant placement in four groups in one side (from left to right: TIC, TIS, SIS, and SIC)

a series of animal studies indicated the inflammatory cells could accumulate near the IAI, and subcrestal placement of IAI induced more inflammatory cells accumulation than that of crestal or supracrestal placement.[5–7] Bacterial challenge is the initiative factor for peri-implant inflammation,[8] resulting in hard and soft tissue damage clinically. However, to the best of our knowledge, studies so far have not compared the microbes around implants placed at different levels, and their correlation with corresponding clinical conditions. A difference at species level has been identified between human and dogs in periodontitis,[9] however, so far as we know, the comparison of species associated with dental implants in dogs and humans remains to be investigated. The common periodontal pathogens, *P. gingivalis*, *T. denticola*, *T. forsythia*, *F. nucleatum*, *P. intermedia*, and *A. actinomycetemcomitans*, were screened as potential peri-implant pathogen candidates by PCR or RT-PCR in humans.[10,11] Therefore, they were selected as target bacteria for evaluation instead in this study.

It has been shown that periodontal pathogenic bacteria could be detected inside the failed implants *in vitro* and *in vivo*, and scholars inferred that bacteria could penetrate from the IAI, resulting in peri-implant soft tissue inflammation and bone resorption.[12–14] The microgap size could influence the infiltration of bacteria through the IAI *in vitro*.[15] The conical connection is one type of implant abutment connection with smaller dimension of IAI. A marginal gap size of ∼1–2 $\mu$m was found for the traditional screwed-in conical connection implants, and this connection design uses a Morse taper to act as a barrier against microleakage and bacterial colonization of the internal implant spaces. Unfortunately, the tightening torque of the screw can create a misfit between the connection components, creating a gap that permits leakage.[16] Another connection design of tapped-in locking-taper IAI has been introduced to resist such microleakage, and this connection is designed with a marginal gap size of 0.5 $\mu$m. The locking-taper IAI will create a hermetic seal as a result of cold welding that occurs between the abutment and implant, irrespective of whether or not a complete seal is formed, and this effect has been proved by *in vitro* studies.[15,17] However, it remains to be elucidated that the effect of these two implant–abutment connection designs with different microgap sizes on peri-implant bacterial colonization *in vivo*.

Hence, the present study was designed to compare the peri-implant microbial colonization of inflamed implants placed at different levels in an animal model and correlate it with its corresponding clinical parameters. The null hypothesis was that the peri-implant microbial colonization of inflamed implants placed at different levels was comparable in dogs.

## 1 | MATERIALS AND METHODS

### 1.1 | Animals

The study was approved by the Medical Ethical Committee for Animal Investigations of Peking University Health Science Center in Beijing, China (approval No. LA2010-032). Six beagle dogs (Beagle dog, Beijing Marshall Biotechnology Co. Ltd., Beijing, China) (aged 1 to 2 years, weighing 10 to 12.5 kg) were included in the study, and the dogs were fed once per day with soft-food diet (Laboratory animals facilities permit: SYXK (Beijing) 2008-0021) during the experiment.

### 1.2 | Implant placement

A split-mouth design was used in the animal study, and the flowchart is presented in Figure 1. All surgical procedures had been described by Huang et al.[18] Briefly, the 2nd, 3rd, 4th premolar and the 1st molar (P2-M1) in the mandible were extracted bilaterally from six dogs with minimal trauma. Scaling was performed to remove supragingival calculus around remaining teeth 1 week before the implant surgery. After 8 weeks healing period, two implants with tapped-in (TI) tapered internal IAI (Integra-CP, 3.5 · 8 mm, Bicon Dental Implants, Boston, MA) and two implants with screwed-in (SI) tapered internal IAI (OsseoSpeed, 3.5 · 8 mm, Astra Tech Dental, Mölndal, Sweden) were inserted at the extraction sites of both sides respectively, and the two implants of the same system were placed at different levels: one crestally, and the other one 1.5 mm subcrestally. Therefore, the implants were grouped into four according to implant system and placement depth: SI implants placed crestally (SIC); SI implants placed 1.5 mm subcrestally (SIS); TI implants placed crestally (TIC); TI implants placed 1.5 mm subcrestally (TIS) (shown in Figure 2) ($N = 8$ per animal, $N = 48$ total). The anterior and posterior distribution of SI and TI implants was alternated to minimize bias. Additionally, the order of placement depths, crestally or subcrestally, within the same implant system was

distributed randomly by toss. All implants were left to heal in a submerged position for 12 weeks, and the second-stage surgery was conducted to replace cover screws with healing abutments afterwards.

Oral hygiene measures were started immediately after abutment exposure. The exposed abutments and peri-implant soft tissues were irrigated with 0.12% chlorhexidine gel at the interval of two days for the first 10 days. After that, an oral hygiene procedure using a soft toothbrush with 0.2% chlorhexidine gel was performed at the same interval.

## 1.3 | Experimental peri-implantitis

Experimental peri-implantitis was induced 4 weeks after abutment connection at the experimental side of each dog chosen at random. Cotton ligatures were placed at a submarginal position around the abutments to promote plaque accumulation and induce plaque-associated peri-implant inflammation.[19] The ligatures were checked once per week to confirm their proper position as related to abutments. Plaque control measures were maintained at the control side and terminated on the experimental side. Plaque accumulation was allowed to continue for 12 weeks.

## 1.4 | Sampling and clinical evaluation

Sampling and clinical evaluations were performed 4 weeks, 10 weeks, and 16 weeks after the second-stage surgery. Samples of peri-implant sulcular fluid (PISF) were collected as described. Sampling sites were isolated from moisture after supramucosal deposits were removed, and filter strips (filter strip, Whatman Co., UK) (2 mm × 10 mm) were inserted into the bottom of the peri-implant sulcus with a mild resistance at the buccomesial and buccodistal aspects of peri-implant sulcus respectively for 30 seconds. Strips were weighted before and after sampling using an analytical balance with a sensitivity of 0.01 mg (AE 240s, Mettler, Zurich, Switzerland) in an air-tight room, and the weight differences were calculated. All strips were stored at -80°C before further process. Clinical measurements, including probing depth (PD) and clinical attachment level (CAL), were also recorded just after sampling.[18] Briefly, PD was measured as the distance from the mucosal margin to the bottom of the sulcus and CAL was measured as the distance from the fixed point in the abutment shoulder to the bottom of the sulcus using a periodontal probe at buccomesial and buccodistal aspects of each implant. All measurements of clinical values were adjusted to the nearest 0.5 mm.

## 1.5 | Management of samples

Strips were washed with TE buffer (10 mM Tris–HCl, pH 7.6, 1 mM EDTA) at a volume of 99 times of volume-weight differences ratio (μL/mg), and the bacterial genomic DNA in the samples was extracted following the manufacturer's guidelines (TIANamp Micro DNA Kit, TIANGEN BIOTECH (BEIJING) CO., LTD., Beijing, China). The bacterial genomic DNA was amplified by PCR for the detection of common periodontal pathogens, including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and *Aggregatibacter actinomycetemcomitans*. The primers, reaction systems, and reaction conditions were following that of Ashimoto et al.[20]

*P. gingivalis* and *T. forsythia* in samples were further analyzed quantitatively with real-time PCR. The mixtures and thermal cycling sequences were used as previously described by Morillo et al.,[21] and the primers were CCTACGTGTACGGACAGAGCTATA (*P. gingivalis* forward), AGGATCGCTCAGCGTAGCATT (*P. gingivalis* reverse), TCCCAAAGACGCGGATATCA (*T. forsythia* forward), and ACGGTCGCGATGTCATTGT (*T. forsythia* reverse). The standard curve was generated with standard ten-times series diluted bacterial reference strains *P. gingivalis* (ATCC 33277) and *T. forsythia* (ATCC 43037) respectively. *P. gingivalis* and *T. forsythia* copies in PISF samples were identified according to the standard curve. All samples were amplified in triplicate.

## 1.6 | Statistical analysis

Standard error of measurement (SE) and Spearman correlation coefficient (CC) for clinical (SE = 0.31 mm; CC = 0.889) measurements were calculated to determine intraexaminer reliability. The mean values and standard deviations were calculated for all the parameters. Experimental data were examined with the Shapiro – Wilk test for normal distribution. If data were not distributed normally, the Wilcoxon test was used to examine the difference between groups; otherwise a paired $t$-test was performed. The null hypothesis was rejected at $P \leq 0.05$. Correlation between *T. forsythia* detection and clinical parameters were analyzed. All statistical analyses were performed using statistical software SPSS (SPSS v.17.0, IBM, Chicago, IL).

## 2 | RESULTS

During the study, healing was achieved around all implants uneventfully. Clinical healthy peri-implant mucosa was also observed around all implants at baseline. Plaque accumulated after ligature placement around 24 implants and marked signs of inflammation were observed around these implants.

## 2.1 | Microbial results

A total of 6 periodontal pathogens were investigated in the peri-implant sulcular fluids, and only *T. forsythia* and

*P. gingivalis* were detected high enough to be analyzed. *P. intermedia* and *A. actinomycetemcomitans* were not detected in all samples. *F. nucleatum* was detected in a quite low rate, and *T. denticola* was detected in only one sample, therefore they were not included into the analysis. The detection rates of *T. forsythia* and *P. gingivalis* at the experimental sides increased significantly after ligature placement in four groups or in total (Table 1). The *T. forsythia* level in PISF was summarized in Table 2. Longitudinally, *T. forsythia* level in the PISF increased significantly from baseline in all four groups at the experimental sides at the time-point of 16 weeks ($p < 0.05$). Additionally, comparison was made among four groups at 16 weeks after restoration at the experimental sides, and only the TIS group harbored significantly more *T. forsythia* than TIC at the experimental sides ($p < 0.05$) (Figure 3). *P. gingivalis* level in PISF was too low to be analyzed by quantitative PCR (data not shown here).

## 2.2 | Correlation between microbial results and clinical parameters

Clinical parameters were correlated to periodontal pathogenic bacteria *T. forsythia* level in the experimental sides and control sides. In experimental sides, PD was found to be correlated to *T. forsythia* level in the four groups as well as in the experimental sides total (Table 3). However, no correlation was found between CAL and *T. forsythia*.

## 3 | DISCUSSION

The present study was designed to evaluate the influence of placement depth on peri-implant microbiology in the early stage of peri-implantitis development in a canine model and correlated it with clinical parameters. After ligature placement, peri-implant lesion, deep pocketing, suppuration, and loss of alveolar bone, was established as defined by Mombelli et al.[8] Based on the results of this study, the null hypothesis was rejected. The TIS group harbored significantly more *T. forsythia* than TIC group at the ligature-induced peri-implant inflammation 16 weeks after restoration ($p < 0.05$).

In the present study, peri-implant infection was induced by ligature placement, and this ligature-induced peri-implant infection animal model was widely applied in studying the pathogenesis and treatment of peri-implantitis.[22–24] After ligature placement, subgingival bacterial accumulation led to peri-implant soft tissue inflammation and subsequently bone loss. Additionally, ligatures were kept in position during the development of inflammation. This ligature placement protocol was comparable to the conventional model in which a twelve-week healing time was allowed.[24] Such early stage models could be useful when the pathogenesis

**TABLE 1** The detection rates of *T. forsythia* and *P. gingivalis* around ligatured or non-ligatured implants in four groups ($N = 6$ per group, %)

| Bacteria | Ligature | Group | 4 Weeks | 10 Weeks | 16 Weeks |
|---|---|---|---|---|---|
| *T. forsythia* | Ligatured | SIC | 0 | 83.33 | 83.33 |
| | | SIS | 0 | 66.67 | 83.33 |
| | | TIC | 0 | 50 | 100 |
| | | TIS | 16.67 | 66.67 | 100 |
| | | Total (N = 24) | 4.17 | 66.67 | 91.67 |
| | Non-ligatured | SIC | 0 | 16.67 | 16.67 |
| | | SIS | 16.67 | 16.67 | 33.33 |
| | | TIC | 0 | 0 | 66.67 |
| | | TIS | 0 | 33.33 | 50 |
| | | Total (N = 24) | 4.17 | 16.67 | 41.67 |
| *P. gingivalis* | Ligatured | SIC | 33.33 | 83.33 | 83.33 |
| | | SIS | 16.67 | 66.67 | 100 |
| | | TIC | 33.33 | 66.67 | 83.33 |
| | | TIS | 50 | 83.33 | 100 |
| | | Total (N = 24) | 33.33 | 75 | 91.67 |
| | Non-ligatured | SIC | 16.67 | 66.67 | 66.67 |
| | | SIS | 33.33 | 16.67 | 66.67 |
| | | TIC | 50 | 50 | 50 |
| | | TIS | 33.33 | 66.67 | 50 |
| | | Total (N = 24) | 33.33 | 50 | 58.33 |

of peri-implantitis was evaluated. Another animal model has been reported to mimic the spontaneous progression of peri-implantitis, in which the ligature was removed after the establishment of peri-implantitis.[25,26] This provides a valuable model to investigate the progression of peri-implantitis into a more advanced stage; however, unpredictable results might form after the ligature is removed in this case, leading to either further progression or a resting lesion.[27,28] In the present study, the ligature was kept in contact with the peri-implant tissue during the whole experimental period, so as to evaluate bacterial accumulation at the early stage and the peri-implant tissues reaction to such accumulation.

Like in periodontitis, microbiota constitutes the initiative factor of peri-implantitis.[29] After exposure in the oral cavity, a complex submucosal microbiota around implants could form in peri-implant sulcus within 2 weeks, and such microbiota could reach a stasis within 3 months.[30] Hence, the experiment time for plaque accumulation was set at 12 weeks, and the study was mainly focused on the early colonization of peri-implant bacteria. In the present study, only *T. forsythia* and *P. gingivalis* were detected at high rates,

**TABLE 2** *T. forsythia* level at different time points around ligatured or non-ligatured implants analyzed by real-time PCR (N = 6 per group, Copy/ log10)

| Ligature | Group | 4 Weeks | 10 Weeks | 16 Weeks | *P* |
|---|---|---|---|---|---|
| Ligatured | SIC | 4.70±0.30 | 6.87±0.77 | 6.10±0.53 | *, †, ‡ |
| | SIS | 4.30±0.77 | 6.23±0.91 | 6.20±0.82 | *, †, ‡ |
| | TIC | 4.63±0.37 | 5.82±0.93 | 6.14±0.19 | *, †, ‡ |
| | TIS | 4.52±1.05 | 5.83±0.94 | 6.61±0.45 | *, †, ‡ |
| | Total (N = 24) | 4.54±0.66 | 6.19±0.94 | 6.26±0.55 | *, †, ‡ |
| Non-ligatured | SIC | 4.32±0.56 | 5.31±0.46 | 5.28±1.02 | NS |
| | SIS | 4.65±0.78 | 4.55±0.58 | 5.03±0.76 | NS |
| | TIC | 4.26±0.69 | 4.69±0.30 | 5.08±0.56 | NS |
| | TIS | 4.52±0.31 | 5.16±0.65 | 5.20±0.97 | NS |
| | Total (N = 24) | 4.44±0.59 | 4.93±0.58 | 5.15±0.80 | *, †, ‡ |

*ANOVA test, $p < 0.05$; †4 week compared to 10 week, $p < 0.05$; ‡4 week compared to 16 week, $p < 0.05$; NS, $p > 0.05$
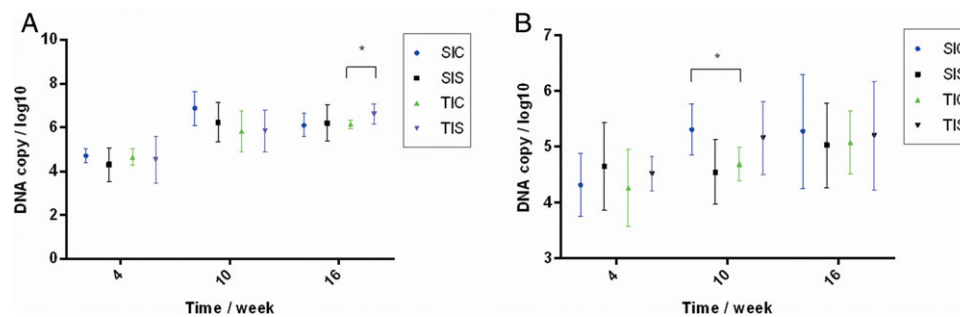


**FIGURE 3** *T. forsythia* level in four groups by Real-time PCR (Copy/log10). **A)** *Tf* detection of ligatured implants; **B)** *Tf* detection of non-ligatured implants. *Significant difference was found ($p < 0.05$)

**TABLE 3** Correlation between *T. forsythia* level and clinical parameters in the experimental sides (R, p)

| | PD | | CAL | |
|---|---|---|---|---|
| Group | R | p | R | p |
| SIC | **0.488*** | **0.020** | .157 | .767 |
| SIS | **0.641** | **0.004** | −.456 | .363 |
| TIC | **0.539** | **0.021** | .158 | .765 |
| TIS | **0.644** | **0.004** | .070 | .895 |
| Total | **0.494** | **<0.001** | −.137 | .524 |

*Correlation was detected in items bolded.

however, only *T. forsythia* has been identified at high levels by quantitative PCR, and *P. gingivalis* was identified at very low levels (data not shown here). Predominant bacterial flora in dogs used for experimental periodontitis and peri-implantitis were identified and cross-reacted with corresponding human species.[9] From cross-reactions, only *T. forsythia* species from dogs' bacterial flora had strong cross-reaction with its corresponding human species among all periodontal pathogenic pathogens. Therefore, only T. *forsythia* was detected high in the samples. *Porphyromonas gulae* has been identified as the most common *Porphyromonas* species in the predominant bacterial flora in dogs, and it shares the

16S rDNA sequences with *P. gingivalis* amplified by PCR, however, the sequences amplified by quantitative PCR varied for the two species, resulting in the discrepancy of *P. gingivalis* amplification results between PCR and quantitative PCR.

In the present study, the detection rate and bacterial counts of periodontal pathogens around implants in the experimental sides both showed significant increase after ligature placement and were significantly higher than those of implants in the control group after ligature placement. These results were consistent with other studies,[31,32] indicating the association between peri-implant tissue inflammation and bacterial accumulation.

Microgap size at the IAI is one factor associated with the peri-implant tissue,[33,34] however, it has exhibited little influence on peri-implant microbial colonization. In the present study, two conical connection systems were used to compare the peri-implant bacteria load, and despite the different microgap sizes, the *T. forsythia* level of these two implants systems was comparable, indicating the small size of the microgap did not hinder the peri-implant bacterial accumulation of ligatured implants.

Peri-implant bacteria were also compared between implants with different placement level. In the present study,

it has been found that the subcrestally placed tapped-in implants harbored more *T. forsythia* than crestally placed ones when peri-implantitis was present. Previous studies by our research group have compared the soft and hard tissue alteration of implants with different placement depths after ligature-induced peri-implant inflammation, and tissue destruction in subcrestal implants was more serious than that of crestal implants in the presence of peri-implant inflammation. In subcrestally placed implants, the peri-implant sulcus probing depth was larger than that of crestally placed ones after ligature. The increased soft tissue sulcus depth resulted in an environment more favorable to apical accumulation of anaerobic periodontal pathogens, for example, *T. forsythia*.

Implants were often placed subcrestally, especially for esthetic reasons. Although the study was designed to evaluate the peri-implant microbiota at early stage, it provided the suggestion that when IAI of implants was placed below the bone level, special attention is necessary to prevent peri-implantitis because implants at this placement depth might harbor more periodontal pathogens in the peri-implant sulcus, resulting in severe soft and hard tissue damage.

Several studies had confirmed that the periodontal clinical parameters are strongly associated with periodontal microbes,[35–37] and such association existed in peri-implant tissue too. A clinical study had shown that peri-implantitis is more likely occurring in deep sulci compared with shallow sulci in partial edentulous patients.[38] In the present study, correlation between clinical parameters and bacterial levels was analyzed, and *T.forsythia* concentration around implants was found to be correlated with peri-implant probing PD and not correlated with CAL in this ligature-induced peri-implant lesion model. Our results are consistent with Dierens et al., in which correlation between bacterial DNA counts around implants and interproximal probing depth or interproximal bleeding index was present.[39] Another study investigated the correlation of the presence of *A. actinomycetemcomitans*, *P. gingivalis,* and *T. forsythia*, three common species detected in peri-implant disease, with the inflammatory degree of the tissues, showing no correlation between bacterial levels and degree of histologic inflammation. The correlation in the present study revealed the association between *T. forsythia* and PD at the early stage of peri-implant disease progression. Peri-implant disease is a disease defined by bacterial infection[8], mainly anaerobic bacteria.[31,40–42] Deep pockets provide a suitable anaerobic reservoir for those microbes, forming the biofilm around implants. Bacterial invasion is the initiative factor of peri-implant disease and reducing peri-implant bacterial level is crucial and inevitable in all peri-implant disease treatment. Because the microbial characteristics are usually not easily available during clinical practice, PD values could be a potential risk predictor in evaluating disease severity because of the association with *T. forsythia* levels. Other than PD, in our previous study, the subcrestal placement of implants showed more bone loss by radiology and histology after ligature, indicating the probable relation between periodontal pathogen colonization and soft tissue and hard tissue change.[26,43] Correlation was also analyzed around healthy implants, however, no correlation was detected between *T. forsythia* and PD, indicating the irrelevance of bacterial counts and clinical conditions.

## 4 | CONCLUSIONS

Within the limitation of the study, it could be concluded that:

1. Subcrestal placement could increase the peri-implant *T. forsythia* level at the early stage of peri-implantitis progression, irrelevant of implants' microgap size.
2. The *T. forsythia* level in the peri-implant sulcus is associated with peri-implant probing depth.

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