Involvement of Bacteria in the Pathological Changes Before Achilles Tendon Rupture

A Case Series Investigating 16S rDNA in 20 Consecutive Ruptures

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Background: The source of the pathological changes that occur before an acute Achilles tendon rupture (ATR) is not fully understood. Bacterial DNA has previously been detected in samples from ruptured Achilles tendons, suggesting a pathogenic role of bacteria in ATR.

Purpose/Hypothesis: The purpose of this study was to investigate if DNA from bacteria was present in acutely ruptured Achilles tendons. We hypothesized that 20% to 30% of the samples from the rupture site and no samples from healthy tissue would be positive for bacterial DNA.

Study Design: Case series; Level of evidence, 4.

Methods: This study included 20 consecutive patients scheduled for surgical repair of an acute ATR. Tendon biopsy specimens were taken from the rupture site and from the healthy tendon tissue proximal to the rupture to act as a control. Samples were blinded to the technician and analyzed using polymerase chain reaction targeted to the bacterial 16S rDNA gene and Sanger sequencing to identify the bacterial species present. McNemar test for paired proportions was performed to test for statistically significant differences in the number of samples positive for bacterial DNA between the ruptured and control regions of the Achilles tendon.

Results: Of the 20 patients, 1 (5%) had a positive sample with bacterial DNA from the ruptured part of the Achilles tendon. The same patient also had a positive control sample, although with different bacterial DNA. An additional patient had a positive control sample. There was no statistically significant difference in the number of bacterial DNA–positive samples between the ruptured and control regions of the Achilles tendon. The bacteria found (*Staphylococcus* sp, *Micrococcus* sp, and *Staphylococcus epidermidis*) were normal commensal organisms on the human skin.

Conclusion: Bacterial DNA was infrequent in tissue from ruptured Achilles tendons and, if identified, likely was a result of contamination. This suggests that bacteria are not involved in the pathological changes occurring before rupture of the Achilles tendon.

Keywords: Achilles tendon; rupture; surgery; etiology; pathogenesis; risk factor; bacteria; 16S rDNA

Acute Achilles tendon rupture (ATR) is a severe injury that leads to permanent functional deficits and sick leave.[7,17,25] To prevent the injury, knowledge regarding the pathophysiology and risk factors for ATR is essential. Studies have shown that pathological changes occur in the tissue of the Achilles tendon before a rupture.[2,8,14] These pathological changes weaken the tendon, which can result in a rupture

during routine movements. It is not fully understood why these changes occur in some but not all Achilles tendons.

Several risk factors for ATR have been discovered: male sex, genetics, treatment with quinolone antibiotics, treatment with systemic glucocorticoids, severe kidney disease, and type 2 diabetes.[6,12,15,21,22,24] Rolf et al[20] recently demonstrated the presence of bacterial DNA in 25% of the samples from ruptured Achilles tendons. This finding has generated the hypothesis that the presence of bacteria plays a role in the pathological changes that occur in Achilles tendons before a rupture. However, the study by

Rolf et al had limitations in the design and in the interpretation of the results primarily because the control samples were taken in a different country than that where the primary biopsies were performed and from another type of tendon (hamstring tendon). This raises the question of whether bacterial DNA in the ATR samples was nonpathological or contamination. No studies have yet tried to reproduce the results found by Rolf et al.

The present study aimed to investigate whether bacterial DNA is present in acutely ruptured Achilles tendons. It was hypothesized that (1) 20% to 30% of the samples from the ruptured part of Achilles tendon tissue would contain bacterial DNA and (2) none of the control samples from the healthy tissue of the same Achilles tendon would be positive for bacterial DNA.

METHODS

Institutional review board approval was received for the study protocol. This study was conducted as a case series, including only patients who underwent surgery for ATR. ATR was defined as a total ATR for which treatment had been initiated within 14 days after injury. Two biopsy specimens were taken per patient: 1 specimen from 1 of the stumps of the ruptured part of the Achilles tendon and 1 specimen taken from the healthy Achilles tendon tissue proximal to the rupture. The tissue samples were analyzed in relation to surgery with real-time polymerase chain reaction (PCR) targeted to the bacterial 16S rDNA gene.[18] PCR products from positive samples were further analyzed with Sanger sequencing to determine the bacterial species.[13]

Patient Recruitment

Patients were recruited consecutively at the Department of Orthopedic Surgery, Copenhagen University Hospital, between May 2019 and April 2021. Inclusion criteria were age between 18 and 70 years, outpatient clinic visit within 4 days after rupture, total acute ATR, and eligibility for operative treatment. Exclusion criteria were (1) rupture of the Achilles tendon at the insertion of the calcaneus or in the musculotendinous junction of the triceps surae; (2) previous rupture of the same Achilles tendon; (3) previous surgery in the same region as the affected Achilles tendon; (4) clinical signs of infection in the affected region; (5) contraindications for surgery, such as severe atherosclerosis with no palpable pulse in the foot or broken skin in the Achilles region of the injured leg; (6) medical treatment for diabetes; and (7) diagnosis of rheumatoid arthritis.

All patients were included in the outpatient clinic after clinical and ultrasonographic examination. Patients were given oral and written information regarding the trial, after which they had the opportunity to decide whether they would like to participate. The participants of the study provided informed consent.

Procedure for Biopsies

Biopsies were performed by trained orthopaedic consultants (K.W.B., P.H.) in the operating theater during surgery. All operations were performed fully open. The procedure was as follows. Surgical scrub and draping, as well as skin incision and dissection, were performed according to the hospital's guidelines. When the ruptured tendon was identified, a new pack containing a sterile scalpel and a forceps was opened. The instruments were used only for taking the biopsy specimen and did not contact the surrounding tissue. A biopsy specimen approximately 5 mm long, 2 mm broad, and 2 mm deep was taken from 1 of the stumps of the ruptured part of the Achilles tendon. The forceps holding the biopsy specimen was handed to the project manager (A.C.), and the biopsy specimen was placed directly into a sterile container without contacting the operating table or other operation tools. The skin incision was elongated, the tendon stump was pulled distally, and the paratenon was opened at the site of the control biopsy. A new package containing a sterile scalpel and a forceps was then opened. A similar-size biopsy specimen was cut out of the proximal part of the Achilles tendon at the transition zone to the gastrocnemius tendon. The forceps holding the control biopsy specimen was handed to the project manager, and the biopsy specimen was placed directly into a new sterile container without contacting the operating table or other surgical tools. Prophylactic antibiotic treatment was given after the biopsies were performed. The biopsy specimens were transported in the sterile container to a refrigerator (5°C) 30 to 60 minutes after collection. The biopsy specimens were then stored in the refrigerator until they were sent for analysis 0 to 4 days later.

After the biopsy specimens were collected, the patients had additionally biopsies performed for investigating the metabolism in the tendon tissue. The results from these analyses have not been received and will be published in an article focusing on the metabolism in acutely ruptured Achilles tendons.

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Analyses of Biological Material

Bacterial 16S rDNA is composed of highly conserved regions and hypervariable regions. The conserved regions are generally identical in almost all bacteria, but the hypervariable regions vary among bacterial species and can be used for identification. This structure of the 16S rDNA enables detection of bacterial depositions in tissue and identification of the species after subsequent sequencing.[3,5,10]

Blinding of the Analyses

The 2 containers with biopsy specimens for each patient were assigned a randomized number (1 or 2) using an electronic random number generator (https://www.randomizer.org/). These numbers were linked to sample location (ruptured part of the tendon or control) via an electronic key. Only the project manager (A.C.) had access to the key. The microbiologist responsible for the interpretation of the PCR and sequencing (C.M.) did not see the containers with biopsy specimens before analysis and did not have access to the key document. Therefore, the microbiologist was blinded.

DNA Extraction, 16S rDNA PCR, and Sanger Sequencing

All biopsy specimens were analyzed consecutively, and the ruptured tendon and the control biopsy specimens were analyzed in the same setup. DNA extraction was carried out in a dedicated preamplification room separate from any postamplification rooms. Extraction of microbial DNA, including depletion of host DNA, and real-time 16S rDNA PCR were performed using Micro-Dx CE IVD kit (Molzym). Tissue samples were pretreated with proteinase K on a heated shaker for 10 minutes at 56°C and 1000 rpm, as described in the kit. The kit included an internal control (ie, a known DNA template) to indicate correct function of the extraction and the PCR reaction. The real-time PCR assay was carried out using reagents from the Micro-Dx CE IVD kit. The PCR reaction targeted the V3-V4 hypervariable region of the bacterial 16S rDNA (481 bp) gene. The PCR reaction also contained 2 positive controls containing a mixture of *Bacillus subtilis* and *Saccharomyces cerevisiae* DNA at a total concentration of 2 pg/µL (high) and 0.2 pg/µL (low). A negative template control (containing no sample) and a negative extraction control were included in the PCR. The number of cycles in positive samples would also be included in the interpretation. The SYBR green–based real-time PCR was carried out in a LightCycler 480 instrument (Roche) with the following conditions: 95°C for 1 minute, 40 cycles of 95°C for 5 seconds, 55°C for 10 seconds, and 72°C for 25 seconds, followed by a melting curve analysis (70°C-95°C).[23]

Sanger Sequencing and Bacterial Species Identification

Samples with a melting temperature peak between 87°C and 91°C for the 16S product were considered positive. PCR products from positive samples were then purified using the QIAquick PCR purification kit (Qiagen). Universal sequencing primers (included in the Micro-Dx CE IVD kit) for gram-positive and gram-negative bacteria were used for Sanger sequencing performed by Eurofins Genomics. Sanger sequencing results were quality controlled using Sequencing Analysis software (Applied Biosystems). Sequences were then BLASTed to the SepsiTest BLAST database (http://www.sepsitest-blast.de/deindex-html) and the NCBI BLAST 16S rRNA database (Bacteria and Archaea; http://www.ncbi.nlm.nih.gov/BLAST/) to identify the species. Percentage identity cutoffs were >99% and >97% for genus- and species-level classification, respectively. Mixed bacterial chromatograms were analyzed using the RipSeq mixed program (iSento) to resolve the individual sequences.[23]

Statistical Analysis

Patient characteristics were reported descriptively. A McNemar test for paired proportions was performed to test for statistically significant difference in the number of bacterial DNA–positive samples between the ruptured and control regions of the Achilles tendon. The threshold for statistical significance was set at $P < .05$. The statistical analysis was performed using R 4.1.0 (R Foundation for statistical computing, Vienna, Austria).

RESULTS

The study included 20 consecutive patients scheduled for surgical repair of ATR. Patient characteristics and the presence of bacteria are reported in Table 1. The mean ± standard deviation age of the patients was 44.9 ± 9.3 years, 70% of patients were male, and 30% had experienced pain in the Achilles tendon before rupture (Table 1).

Of the 20 samples, 1 (5%) from the ruptured part of the Achilles tendon was positive for bacterial DNA, specifically *Micrococcus* sp. The specific species could not be obtained. This patient also had a sample positive for bacterial DNA in the control region of the Achilles tendon but with *Staphylococcus epidermidis*. Additionally, 1 patient had a control sample positive for bacterial DNA (*Staphylococcus* sp). All remaining samples were negative for bacterial DNA (Table 1). There was no statistically significant difference in the number of bacterial DNA–positive samples between the ruptured and control regions of the Achilles tendon ($P \geq .05$, McNemar test).

Interpretation of the PCR and Sequencing

Essential details from the PCR reaction are described in Table 2. For all 3 positive samples, a melting curve demonstrated a peak between 87°C and 91°C, which was the expected melting temperature for the amplified 16S rDNA sequence. These samples crossed the fluorescence threshold cutoff (quantification cycle [C_q]) after ≤30 cycles of PCR. This can be compared with the low-concentration positive control, which contained approximately 1 pg of DNA and

TABLE 1
Patient Characteristics and Presence of Bacterial DNA

Patient	Age at Rupture, y	Sex	Pain Before Rupture	Achilles Tendinopathy	Prior Corticosteroid Use		Bacterial DNA Present	
					Injections	Systemic Treatment	Ruptured Region	Control Region
1	54	Male	No	No	No	No	No	No
2	49	Male	No	No	No	No	No	Yes[a]
3	50	Male	No	No	No	No	No	No
4	29	Female	No	No	No	No	No	No
5	55	Male	No	No	No	No	No	No
6	26	Female	Yes	No	No	No	No	No
7	38	Male	Yes	No	No	No	No	No
8	52	Male	Yes	No	No	No	No	No
9	53	Female	No	No	No	No	No	No
10	49	Male	No	No	No	No	No	No
11	54	Female	No	No	No	No	No	No
12	44	Male	Yes	No	No	No	Yes[b]	Yes[c]
13	32	Male	No	No	No	No	No	No
14	41	Male	No	No	No	No	No	No
15	51	Female	Yes	Yes	No	No	No	No
16	45	Male	No	No	No	No	No	No
17	40	Female	No	No	No	Yes	No	No
18	38	Male	Yes	No	No	No	No	No
19	59	Male	No	No	No	No	No	No
20	39	Male	No	No	No	No	No	No

[a]*Staphylococcus* sp.
[b]*Micrococcus* sp.
[c]*Staphylococcus epidermidis*.

TABLE 2
Details From the Polymerase Chain Reaction[a]

Patient: Sample	C_q (General Limit <30)	Sample Score (General Limit ≥30)	Species Identity[b]
2: control	30.63	25	*Staphylococcus* sp, 98%
12: ruptured	26	45	*Micrococcus* sp, 99.7%
12: control	26	38	*Staphylococcus epidermidis*, 100%

[a]C_q, quantification cycle.
[b]For species level >99% and genus level >97%.

demonstrated a C_q value of –30. Sanger sequencing generated acceptable quality sequence results for 2 samples, with a mean score ≥30 (Phred quality value of base calls). One control sample displayed a relatively high C_q value as well as a sample score <30, suggesting a lower-quality Sanger result. Two samples were able to be identified at genus-level resolution: *Staphylococcus* sp and *Micrococcus* sp. One sample was identified at the species level as *Staphylococcus epidermidis*.

DISCUSSION

This study examined patients with ATR and identified bacterial DNA in 5% (1 of 20) of tissue samples from the ruptured part of the Achilles tendon and 10% (2 of 20) of tissue samples from the healthy part of the tendon. As such, both our hypotheses were rejected, as <20% of the samples from the ruptured part of Achilles tendon tissue contained bacterial DNA and 2 control samples were positive for bacterial DNA. There was no statistically significant difference in the proportion of positive samples between the regions. The bacteria species identified are normal commensal organisms on the human skin, suggesting that the finding likely is a result of contamination.[4] Therefore, the results from the present study suggest that bacteria are not involved in the pathological changes that occur in the Achilles tendon before a rupture.

The results from the present study contrast with those of Rolf et al,[20] who found bacterial DNA in 25% (5 of 20) of the ruptured Achilles tendons while none of the control samples were positive. Given this finding, they suggested a potential involvement of bacteria in the pathogenesis of ATRs. However, as mentioned in the introduction, the study by Rolf et al had limitations in the design and the interpretation of the results. First, the control biopsy

samples were from hamstring tendon grafts used in anterior cruciate ligament reconstruction. Using control samples from another type of tendon taken during a different surgical procedure might result in different risks of contamination. Second, none of the control samples were from the same patient as the biopsy specimen of the ruptured part. Furthermore, 17 of the 23 control biopsies were performed in Hong Kong, while all biopsies of the ruptured Achilles tendons were performed in Sweden. National differences in aseptic handling procedures during surgery and later handling of the biopsy specimens may exist, which could result in a difference in the risk of contamination.[9,16] Third, it was not evaluated individually for each patient whether a positive sample in the ruptured Achilles tendon was most likely due to contamination or was a sign of in vivo infection. Bacterial species were determined in only 3 of 5 positive samples. In the 3 samples, the predominant species identified belonged to the genus *Staphylococcus*. *Staphylococcus* spp are the most abundant organisms of the skin microbiome in the adjacent anatomic region of the Achilles tendon (popliteal fossa and heel).[11] The finding of *Staphylococcus* spp in the positive samples from the Achilles tendon tissue increased the likelihood of the samples being positive due to contamination.

In contrast to the study by Rolf et al,[20] the present study used control samples taken from the same tendon during the same operation. This design results in a close-to-identical risk of contamination for the samples from the ruptured part of the tendon and the controls. A nearly identical risk of contamination minimizes the risk of bias. Furthermore, the likelihood of contamination for the results of the positive samples was evaluated for each patient by a microbiologist blinded to whether the sample was from the ruptured tendon or the control.

The bacterial species in the 3 positive samples in the present study are considered low pathogenic species, especially the *Micrococcus* sp. Where coagulase-negative staphylococci or micrococci were reported in human disease, the cases involved implant of a foreign body or a bloodstream access device or similar device.[1,19] A direct pathogenic role of the *Micrococcus* sp in a native ATR is highly unlikely.

Limitations

The present study was limited by our taking only 1 biopsy specimen from the ruptured part of the tendon and thereby investigating just a small part of the ruptured tendon fibers. As such, if bacterial DNA was exclusively present in other parts of the ruptured tendon, it would not have been identified. Yet, degenerative changes are found in all biopsy specimens from the ruptured part of the Achilles tendons.[14] Therefore, it is considered more likely that the potential presence of bacteria is widespread in the ruptured area and not in a patchy distribution in the tendon. Another limitation was the control samples taken proximally from the ruptured tendon. One could argue that the whole tendon was affected. The transition zone between ruptured tissue and healthy tissue might extend proximally into the area where the control biopsy specimen was taken. Still, one would expect to detect DNA from identical species in

the ruptured part and the unaffected area. Finally, it was a limitation that the sensitivity and specificity have not been evaluated for detecting bacteria in tendon tissue with 16S rDNA PCR. However, the method has shown high sensitivity (92.5%) and specificity (95.7%) for diagnosing bone and joint infections.[10]

CONCLUSION

Bacterial DNA is infrequent in tissue from ruptured Achilles tendons and, if identified, is likely a result of contamination. The results from the present study suggest that bacteria are not involved in the pathological changes that occur in the native Achilles tendons before a rupture. Other causes need to be identified to understand the pathogenesis of ATR.

ACKNOWLEDGMENT

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