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Original Article

Cultivation of *Enterobacter Hormaechei* from Human Atherosclerotic Tissue

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***Aim*: To determine whether culturable bacterial strains are present in human atheromatous tissue and to investigate their properties using culture, quantitative PCR, metagenomic screening, genomic and biochemical methods.**
***Methods*: We analyzed femoral atherosclerotic plaque and five pairs of diseased and healthy arterial tissue for the presence of culturable bacteria using cell cultures and genomic analysis.**
***Results*: Gram negative aerobic bacilli were cultivated from the plaque tissue. Ribosomal 16S DNA amplification and sequencing identified the isolates as *Enterobacter hormaechei*. The isolate was resistant to ampicillin, cefazolin, and erythromycin. A circular 10kb plasmid was isolated from the strain. Antibiotic protection assays of the isolate demonstrated invasive ability in a human monocytic cell line. To extend the study, five matched pairs of diseased and healthy aortic tissue were analyzed via quantitative PCR. Eubacterial 16S rDNA was detected in all specimens, however, *E. hormaechei* DNA was detected in surprisingly high numbers in two of the diseased tissues only.**
***Conclusions*: While it is well documented that inflammation is an important risk factor for vascular pathophysiology, the association of bacteria with atherosclerosis has not been clearly established, in large part due to the inability to isolate live bacteria from atheromatous tissue. This is the first study providing direct evidence of *Enterobacter* spp. associated with atheromatous tissues. The data suggest that chronic infection with bacteria may be an under-reported etiologic factor in vascular pathogenesis. Importantly, characterization of the clinical isolate supports a model of atherogenesis where systemic dissemination of bacteria to atherosclerotic sites may occur via internalization in phagocytic cells.**

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Key words; Atherosclerosis, Atheroma, Bacteria, Invasion, *Enterobacter hormaechei*

Introduction

Atherosclerosis (AS), the leading cause of death in the United States, is a chronic local inflammatory disease of medium and large-size arteries. Inflammatory processes are involved at every stage of the disease, from initiation to progression to acute ischemic events. There is ample epidemiological evidence regarding the association of bacterial infections with atherosclerotic inflammations[1)]. C-reactive protein has been recognized as a "risk factor" for AS development[2)]. Recently, the association of Chlamydia pneumoniae infection with the risk of mortality from coronary heart disease in Japanese women was found[3)]; however, the nature of the causative inflammatory agents has not been adequately addressed. Since many cardiovascular disease (CVD) patients do not have any of the established risk factors (hypercholesterolemia, hypertension, diabetes, smoking, and genetic factors)[4)], the focus of our investigations is to determine

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whether bacterial pathogens may be found at sites of vascular inflammation.

Ribosomal 16S rDNA analysis is a valuable molecular tool for bacterial identification, especially in the case of unculturable bacteria and culture-negative specimens. Based on quantitative PCR (Q-PCR) analysis of atheroma DNA for the presence of specific pathogens, we demonstrated bacterial 16S rDNA representing ten species in atheromatous plaques from both young (mean age 21) and elderly (mean age 67) patients. These data suggest the possibility of a polymicrobial origin for atherogenesis[5]. Similarly, 16S PCR identification of bacterial DNA in atheromas revealed the presence of DNA from 17 different bacterial genera[6]; however, only the culture and characterization of viable clinical isolates from diseased tissue can identify the causative role of bacterial infection in atherosclerotic inflammation.

Notably, there have been attempts to isolate and identify bacterial pathogens directly associated with atheromatous tissue. Most of the studies have ended without success, probably due to the inability to cultivate the bacteria *in vitro*[7]. To date only a few viable organisms, such as Chlamydophila (Chlamydia) pneumoniae[8] and periodontal pathogens[9], have been found in vascular lesions. In this study, our aim was to culture, identify and characterize clinical bacterial strains from atheromatous tissue using a tissue culture approach, which resulted in the isolation of an *Enterobacter* species, *E. hormaechei*[10]. *Enterobacter* spp. are opportunistic pathogens which can cause bacteremia and have been associated with soft tissue and systemic infections, including endocarditis and meningitis. To our knowledge this is the first report associating *Enterobacter* spp. with atheromatous lesions. Following this, *Enterobacter* spp. DNA corresponding to a large number of bacteria was also detected in diseased vascular tissues but not in healthy matched controls. Finally, we describe the interactions of the clinical isolate with different host cell types that suggest a mechanistic model of the hematogenous dissemination of bacterial pathogens. The model may explain our findings and involves systemic infection where the key step is the persistence of intracellular bacteria in phagocytes.

Aim: To determine whether culturable bacterial strains are present in human atheromatous tissue and to investigate their properties using vascular specimens, tissue cultures, genomic and biochemical methods.

Methods

Human Specimens

An atherosclerotic plaque tissue specimen was obtained from a 78-year old male with hypertension and prior myocardial infarction who was admitted with a septic left hydroureter and an infected left aortoiliac prosthetic bypass. On exploration there was erosion of the midportion of the left iliac Dacron limb into the sigmoid colon with an infected clot in a 6 cm hypogastric aneurysm; both femoral arteries were patent but atherosclerotic. Surgical management included endoaneurysmorrhaphy and removal of a very dense and calcified atherosclerotic plaque specimen from the femoral artery. Intraoperative cultures were negative, except for Candida albicans. In addition, five samples of aortic tissue were obtained post-mortem from five individuals; none of the samples was associated with sepsis. In each post-mortem, tissue areas were selected as 'healthy' and diseased' by the pathologist, and healthy control tissue was obtained from areas adjacent to the atheroma. Tissue from patients and post-mortem specimens were obtained from the Columbia University Medical Center Department of Surgery and Department of Pathology according to approved Institutional Review Board protocols.

Cell lines, Tissues and Reagents

The *Enterobacter hormaechei* strain isolated from vascular tissue was grown aerobically on Trypticase soy agar (BBL, Cockeysville, MD) supplemented with sheep blood (5%), hemin (5 mg/mL), and menadione (5 mg/mL) or on Luria Bertani (LB) agar plates at 37℃. The type strain *E. hormaechei* ATCC 49162 was purchased from ATCC (Manassas, VA). Liquid cultures were grown in LB broth aerobically.

Caco-2 cells (ATCC HTB-37, a kind gift from Dennis McGee), a colorectal adenocarcinoma cell line, were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and non-essential amino acids.

Human aortic endothelial cells (HAEC) (Cascade Biologics, Carlsbad, CA), a primary cell line, were grown in Endothelial Basal Media-2 (EMB-2) supplemented with EGM-2 SingleQuot supplements and growth factors (Lonza, Basel, Switzerland).

THP-1, a human monocytic cell line (ATCC, Manassas, VA), was maintained in RPMI-1640 medium (MediaTech, Manassas, VA) supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol.

Tissue Processing and Clinical *E. Hormaechei* Strain Cultivation

The femoral plaque was placed post-surgery in saline at 4℃ and processed 18 hours after harvesting, as follows. The specimen (0.5 g) was washed, minced,

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ground and suspended in 5 mL phosphate-buffered saline (PBS) using a glass homogenizer. The suspension was aspirated through a 26-gauge needle and spun down at 1,400Xg for 7 min. The supernatant was plated on blood agar plates (BAP) and incubated anaerobically, while the pellet was lysed with 2 mL sterile water (0.1% Triton X-100) for 20 min at room temperature. The lysate was centrifuged at 16,000Xg for 10 min, the resulting pellet was resuspended in 5.5 mL RPMI-1640 medium and 1 mL of the suspension was added to 1 mL THP-1 cells (5×10^5 cells) for overnight incubation at 37℃, 5% CO_2. The cell suspension was then centrifuged at 1,400Xg for 7 min, the pellet was lysed with 1 mL sterile water (0.1% Triton X-100) for 20 min at room temperature, and the lysate was plated on BAP.

Bacterial Genomic DNA Isolation and 16S rDNA gene Amplification

Genomic DNA from clinical *E. hormaechei* strains was isolated using a DNeasy Tissue kit (Qiagen, Valencia, CA.) using the Purification of Genomic DNA from Gram-Negative Bacteria protocol. A nearly full-length segment of 16S rDNA was amplified using Advantage 2 Polymerase mix (Clontech, Mountain View, CA.) with universal 16S rDNA primers 9F (5'-GAGTTTGATYMTGGCTCAG-3') and 1541R (5'-AAGGAGGTGWTCCARCC-3')[11]. The resulting 1.5 kb PCR fragment was isolated using agarose gel electrophoresis and sequenced on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA.) with the same primers and with an additional internal primer 530F (5'-GTGCCAGCAGCCGCGG-3').

GenBank and Phylogenetic Analysis

The complete sequence of the 16S rDNA segment was assembled in Seqman II (DNAStar, Madison, WI.) and further analyzed using the NCBI and Ribosomal Database Project (RDP) 16S RNA classifier[12] on the Ribosomal Database project website (http://rdp.cme.msu.edu/index.jsp).

DNA Isolation from Tissue Specimens

Genomic DNA was isolated as previously described[5].

Tissue Analysis for *Enterobacter* spp. DNA

We tested total DNA from five pairs of atheromatous and control healthy tissue for the presence of *E. hormaechei* DNA using 16S rDNA gene amplification and universal primers. The primers (EV3F, 5'-TTGACGTTACCCGCAGAAGA-3' and EV4R, 5'-ACCGCTACACCTGGAATTCTAC-3') define a 213 bp amplicon spanning V3 and V4 regions of the 16S RNA sequence of *E. hormaechei* broadly covering the majority of *Enterobacter* species within the *Enterobacteriaceae* family. The primers were designed using Vector NTI software. The concentration of *E. hormaechei* control genomic DNA was determined using the Quant-IT dsDNA HS Assay (Invitrogen, Carlsbad, CA.). A series of 10X dilution calibration samples were used, starting at 500 pg/μL down to 0.5 fg/μL or ~0.16 molar equivalents of 16S rDNA per microliter. Three replicates containing 100 ng of the DNA isolated from the atheromatous samples were amplified using real-time PCR against three replicates of serially diluted *E. hormaechei* calibration samples and six template-free controls.

Two microliters of each sample were added to 18 μL of 1x Platinum SYBR Green QPCR SuperMix-UDG (Invitrogen) containing 200 nM of each primer and amplified for 40 cycles using the ABI 7000 Sequence analysis system with a default protocol. Upon completion of the protocol, the levels of positive signals obtained in the PCR with atheromatous samples were compared with the matching healthy tissue samples and tissue-free controls using Student's *t*-test, and the specificity of the amplicons was interrogated with melting curve analysis followed by amplicon size analysis using an Agilent Bioanalyzer and sequencing.

Metagenomic Screening of the Atheromatous Specimens

In addition to the original patient specimen, the same five pairs of human diseased and matching adjacent healthy (D/H) tissue were analyzed for the presence of eubacterial DNA. Bacterial DNA present in the specimens was analyzed using 16S rDNA amplification, as follows. A nearly full-length segment of 16S rDNA was amplified from the genomic DNA templates using Advantage 2 Polymerase mix (Clontech) with universal 16S rDNA primers 9F (5'-GAGTTTGATYMTGGCTCAG-3') and 1541R (5'-AAGGAGGTGWTCCARCC-3')[13]. A "no template control" (NTC) reaction was included in the experimental protocol to monitor for the external contamination of the samples.

Analysis of Plasmid Content

E. hormaechei plasmid DNA was extracted from broth culture using a Qiagen Plasmid Mini Purification kit as described (Qiagen, Valencia, CA). For visualization, field inversion gel electrophoresis (FIGE) was used[14].

Advance Publication
Journal of Atherosclerosis and Thrombosis
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Antibiotic Susceptibility Testing

The antimicrobial susceptibility of the clinical isolate was determined using a VITEK-2 automated system for antibiotic susceptibility testing (bioMerieux, Durham, NC). The following agents were tested: amikacin, ampicillin, ampicillin/sulbactam, aztreonam, cefazolin, cefepime, cefotetan, ceftazidime, ceftriaxone, cefuroxime, cefuroxime axetil, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, nitrofurantoin, piperacillin, piperacillin/tazobactam, tobramycin, trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid and cefotaxime. Advanced Expert System software (BioMerieux) was used in conjunction with the VITEK 2 test system to determine the antimicrobial susceptibility of the isolate and 48 biochemical properties for microbial identification. Erythromycin resistance was determined by agar disk diffusion.

Tissue Attachment and Invasion

The tissue attachment and invasion ability of the isolate was tested *in vitro* on the following human cell lines: THP-1 monocytic cell line, Caco-2 intestinal epithelial and HAEC-1 endothelial cell line. An antibiotic protection assay was used essentially as described[15], with modifications as follows.

For the antibiotic protection assay, an overnight broth culture of *E. hormaechei* was quantitated using OD600 and diluted to 10^7 colony-forming units (cfu) per ml in the appropriate tissue culture medium. One milliliter of *E. hormaechei* inoculum was overlayed on the target cells (MOI 100), which were incubated for 90 minutes at 37℃, 5% CO_2 and washed once with PBS. When using a suspension THP-1 cell line, 10^5 cells/well were seeded just prior to invasion. As an invasion control, *E. coli* strain MC1061 was used. To determine the exact number of bacteria added to the tissue culture cells, dilutions of the inoculum were plated.

For attachment assays, after the 90-minute invasion, the cells were washed and lysed with 1 mL sterile water for 20 minutes with rocking at 4℃. Lysates were diluted accordingly and plated on LB agar in triplicate. After 24-hour incubation at 37℃, plates were counted to determine the cfu/mL of attached and invaded bacteria.

For invasion assays, after the 90-minute invasion, the cells were incubated with tissue culture medium containing 300 μg/mL gentamicin and 400 μg/mL metronidazole for 1 hour at 37℃, 5% CO_2 to kill extracellular bacteria. After antibiotic treatment, cells were washed three times with PBS and lysed with 1 mL sterile water for 20 minutes with rocking at 4℃. Lysates were diluted accordingly and plated on LB agar in triplicate. After 24-hour incubation at 37℃, colonies were enumerated to determine the cfu/mL of invaded bacteria. The experiments were performed in triplicate. The resulting cfu counts were normalized to 10^7 bacteria used for individual well inoculation and the data was analyzed using Student's *t*-test.

Persistence in THP-1

For extended intracellular survival (persistence), the invasion assay was carried out as described in the above protocol, with the following modifications for suspension host cells. After washing, the cells were resuspended in antibiotic-containing medium and incubated for either 24 or 48 hours at 37℃, 5% CO_2. THP-1 cells were then washed three times with PBS, lysed with 1 mL sterile water for 20 minutes, lysates were diluted accordingly and plated on LB agar in triplicate. After 24-hour incubation at 37℃, colonies were enumerated to determine the cfu per ml of intracellular bacteria.

Results

Cultivation of Clinical Isolates from Atheromatous Tissue and Identification of *E. Hormaechei*

Five bacterial colonies were cultivated from the septic patient tissue (four via THP-1 and one from direct plating). Colony morphology on solid medium was smooth, typical of *Enterobacteriaciae*. Gram staining identified the isolates as Gram-negative rods (**Fig. 1**). Control VITEK 2 automated microbiology system with Advanced Expert software identified the isolate as belonging to *Enterobacter* spp. Forty-seven established biochemical tests were used as well as newly developed substrates measuring carbon source utilization, enzymatic activities, and resistance. Further phylogenetic assignment of the cultivated species was performed via 16S rDNA amplification on a bacterial genomic DNA template. DNA was purified and analyzed from all five initial isolates. As a result of this analysis, the sequence was assigned to the *Enterobacter* genus[16], and specifically to sequence S000544285, which belongs to *Enterobacter hormaechei*[17].

Quantitation of *E. Hormaechei* Genomes in Atherosclerotic Plaques

We used quantitative PCR to determine the presence of bacterial genomic DNA in five matching pairs of diseased and healthy arterial tissue. Statistical analysis of the Q-PCR data followed the standard hypothesis testing framework using corresponding functions implemented in Microsoft Excel. A qPCR calibration

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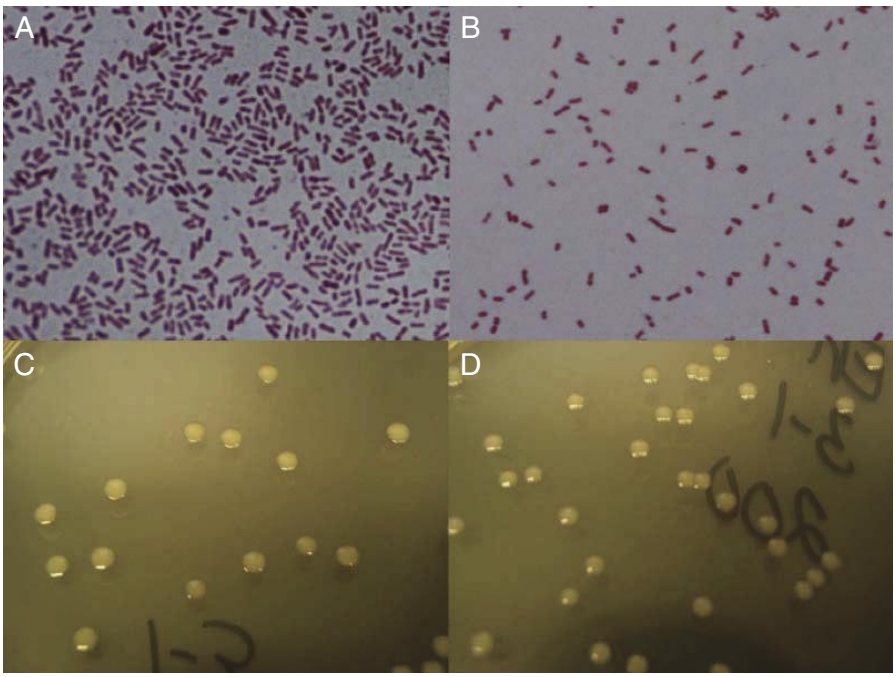

Fig. 1. Gram staining and colony morphology of the clinical isolate (panel A and C) and the type strain *E. hormaechei* ATCC 49162 (panels B and D).

curve was built using triplicate dilutions of *E. hormaechei* DNA obtained from the bacteria isolated from the atheromatous tissue. The average C_t value was assumed to be "undetected" if more than one replicate sample in the group of three demonstrated "undetected" Ct. Differences between no-template controls (NTCs) and the sample replicates were estimated as two-sample *t*-test-associated *p*-values assuming single-tailed distribution and unequal variance. The null hypothesis was rejected at the 0.05 probability level. Analysis of two of the five samples from diseased areas of aortic tissue (D1 and D5) showed the presence of *E. hormaechei* 16S rDNA at statistically significant levels both against NTCs ($p<0.0085$ and $p<0.0008$, respectively) and against their controls obtained from healthy areas. As compared to the calibration curves derived from serial dilutions of the *E. hormaechei* DNA and assuming an *E. hormaechei* genome size of 3 Mb, the average Q-PCR Ct values in samples D1 and D5 corresponded to the presence of 184 and 15 bacterial genomes per 100 ng of total DNA isolated from samples D1 and D5, respectively, with the overall sensitivity threshold of the Q-PCR being about 0.3 genomes per PCR sample.

Metagenomic Screening of the Atheromatous Specimens

Sample pairs (diseased and adjacent healthy postmortem tissue) from five individuals were analyzed for the presence of eubacterial DNA. The results of 16S rDNA PCR using 9F and 1540R primers as they appear after analysis of DNA from the surgical specimen and the matched pairs of diseased and healthy tissue demonstrated the presence of eubacterial DNA in all five tissue pairs. There were no amplification products in the NTC samples. For analysis of amplified DNA, the Agilent Bioanalyzer was used.

Plasmid Presence

A circular 10 kb plasmid was isolated from the clinical *E. hormaechei* isolate and visualized directly and after isothermal strand-displacement amplification (GenomiPhi V2 enzyme mix; GE Healthcare) using field-inversion gel electrophoresis (FIGE) (**Fig. 1**).

Antimicrobial Susceptibility and Biochemical Tests

The isolate was resistant to ampicillin (16 μg/mL), cefazolin (≥64 μg/mL) and erythromycin (300 μg/mL), but was susceptible to the other tested antimicrobial agents (see Methods). Forty-eight biochemical tests were performed to identify the isolate (data not shown).

Antibiotic Protection Assays

The *E. hormaechei* clinical isolate invades THP-1

Advance Publication
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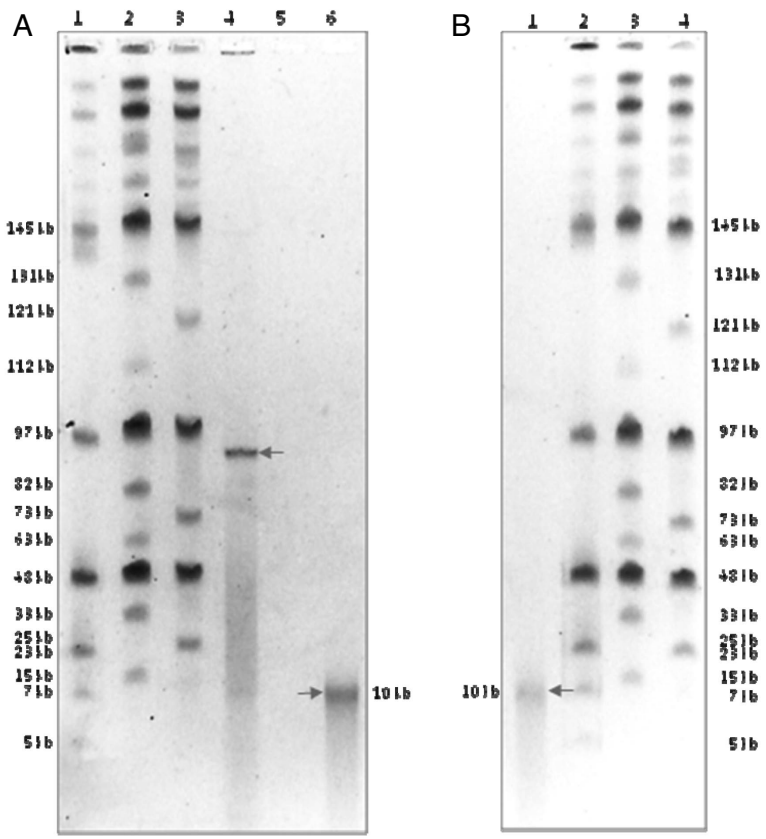

Fig. 2. Field-inversion gel electrophoresis of plasmid from the clinical *E. hormaechei* isolate.

A. Plasmid DNA was linearized with mung-bean nuclease and sized by FIGE. Lanes (1-3): Low range, Mid-range I and Mid-range II PFGE fragment size markers (NEB; Ipswich, MA). Lane 4: Intact plasmid DNA; Lane 6: Linearized plasmid DNA. B. Plasmid DNA was amplified using isothermal strand-displacement amplification (GenomiPhi V2 enzyme mix; GE Healthcare) and sized on FIGE. Lane 1: Amplified plasmid DNA; Lanes 2-4: DNA size markers (NEB). Arrows indicate the plasmid position.

monocytic cells (7.6×10^3 +/− 1.7×10^3, **Fig. 2A**) while it is very adhesive but low invasive to intestinal epithelial cells Caco-2 (4.25×10^5 +/− 3.6×10^5 and 3.7×10^2 +/− 16 attachment and invasion, respectively, **Fig. 2B**) as well as HAEC primary endothelial cells (2.5×10^5 +/− 1.4×10^5 and 242 +/− 173, **Fig. 2C**). *Escherichia coli* MC1061, used as a positive control, had invasion efficiency of 4.2×10^3 +/− 6.2×10^2 for THP-1 monocytes, 2.3×10^4 +/− 1.5×10^4 for epithelial cells, and 1.2×10^4 +/− 4.79×10^3 for endothelial cells. In the persistence experiment controlled with the ATCC-type strain, intracellular persistence of the clinical isolate *E. hormaechei* in monocytes was observed after 1.5 hours (6.27×10^3 +/− 1.86×10^3), 24 hours (5.45×10^3 +/− 1.63×10^3) and 48 hours (3.39×10^3 +/− 3.37×10^3) (**Fig. 3D**). In the same experiment, the persistence of the control type strain *E. hormaechei* ATCC 49162 in the THP-1 host was assessed after 1.5 hours (1.06×10^5), 24 hours (3.71×10^4) and 48 hours (4.2×10^2).

Discussion

Routes of Systemic Infection

To investigate the causative role of pathogens in atherosclerotic inflammation, it is necessary to isolate and identify viable organisms associated with the diseased tissue. In this study, we examined a model of vascular infection where bacterial pathogens were directly associated with the diseased site, and identified then as *Enterobacter hormaechei*. There are at least two avenues that a pathogenic organism can utilize to disseminate to a distant site, colonize and initiate/exacerbate the inflammatory process. First, pathogenic

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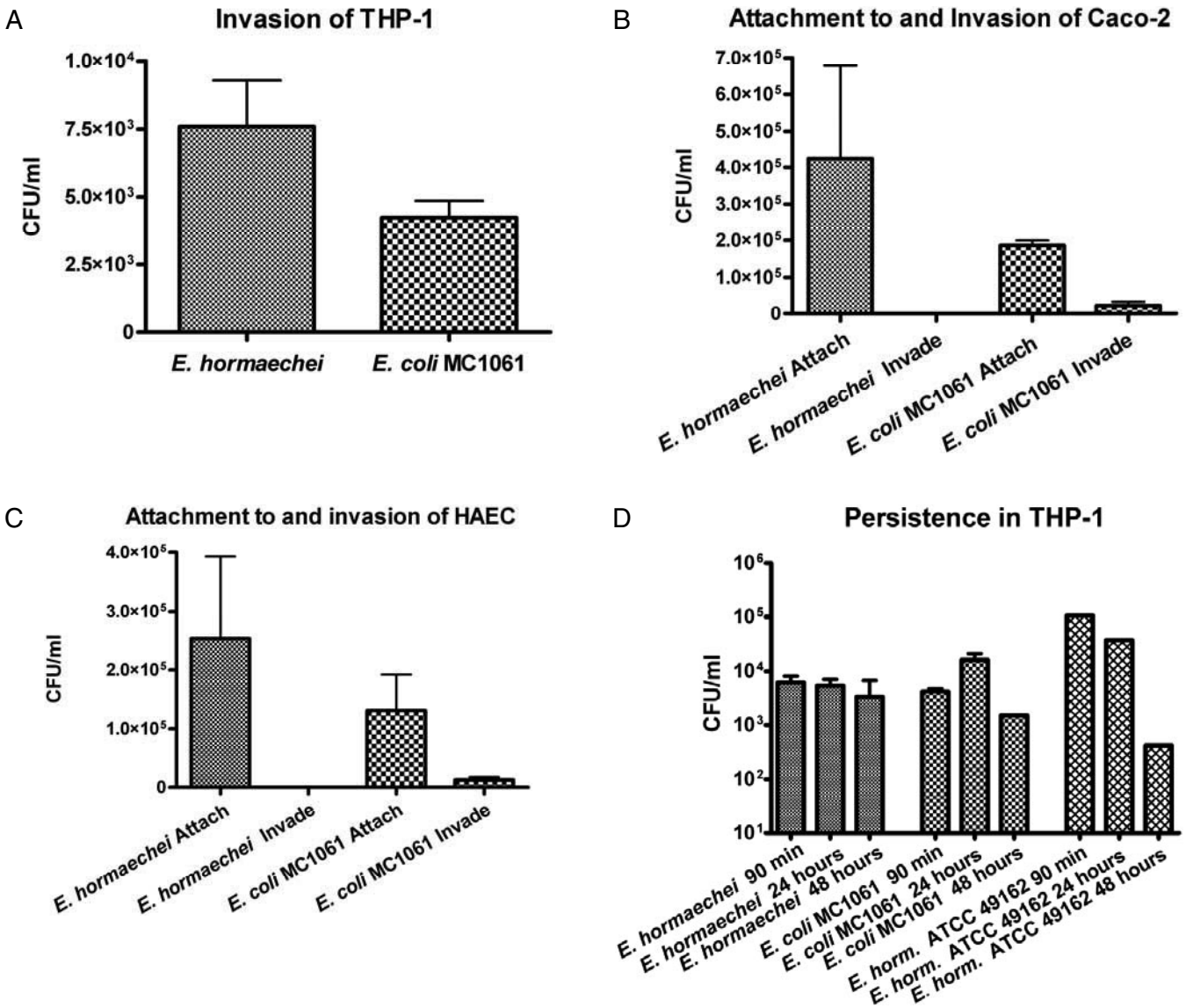

Fig. 3. *E. hormaechei* isolate attachment and invasion of human phagocytic, epithelial and endothelial cell lines.

A, invasion of monocytic cells (THP-1); B, attachment and invasion of intestinal epithelial cell line (Caco-2); C, attachment and invasion of primary endothelial cells (HAEC). D, persistence of the clinical isolate and the type strain *E. hormaechei* ATCC 49162 for 24 and 48 hr in monocytic cells. *E. coli* MC1061was used as positive invasion control. CFU, colony-forming units.

bacteria can gain access to the circulation through different "gates" in the vascular walls and create secondary infections due to affinity (adherence, invasion) to vascular tissue at remote sites. This model, applied to intestinal infections, involves a pathogen that would first gain access to the circulation, either through a transcellular mechanism (invasive bacteria) or paracellularly (transmigrating through the cell-cell junctions or through a breach in the epithelial and endothelial intestinal tissue). The pathogen can then be transmitted rapidly to distant locations where it may attach, colonize and/or invade the tissue, thus initiating or aggravating an inflammatory lesion. This first avenue is less plausible with some organisms, since opsonization and phagocytosis take place quickly; however, another route of dissemination can be via a "Trojan horse" approach, internalization in circulating phagocytic cells and dissemination, provided the pathogen can survive the hostile intracellular environment. The latter route might explain the abundance of bacterial species whose DNA has been identified in atheromatous tissues, including *Enterobacter* spp[18].

Advance Publication
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We have previously shown that DNA from a variety of bacteria can be found at sites of vascular inflammation[5]. Further, we have demonstrated that bacteria are readily transmitted between different primary cell types, which may contribute to the chronic character of the infection[19]. We have also reported the association of bacterial pathogens from periodontal sites with atherosclerotic plaque, which demonstrated that an inoculum of bacteria can enter the circulation and access distant sites using a hematogenous route[9]. Atherosclerosis might be a specific variety of a chronic inflammatory process[20]. Multiple species have been identified in bacteremic blood after dental procedures[11]. Here we present our finding of *Enterobacter hormaechei* isolated from excised plaque tissue obtained from an atherosclerotic patient with sepsis.

Clinical Strain Isolation

To obtain clinical isolates from atheroma, a tissue co-culture approach was utilized as first reported with mycobacteria[21]. We have also observed viable bacteria from atheromatous tissue after their transmission into freshly added tissue culture cells[9]. Similarly, in this study, we used the phagocytic property of the THP-1 monocytic cell line for the isolation of bacteria from atheromatous tissue. Utilizing the tissue culture approach, we isolated multiple colonies of an aerobic organism. Ribosomal DNA analysis identified a single enteric species, *E. hormaechei*.

Clinical *Enterobacter* spp. isolates are associated with pneumonia and sepsis and carry multiple antibiotic resistance and virulence genes[22]. *Enterobacteriaceae* have been implicated in bloodstream infections, in addition to peritonitis, cholangitis, and other intra-abdominal infections[23]. *E. hormaechei* is the species of *E. cloacae* complex most frequently recovered from clinical specimens[17]. In particular, *E. hormaechei* is often associated with nosocomial outbreaks and demonstrates β-lactamase activity[24].

Study of Paired Diseased and Healthy Tissues

Further, to extend our observations beyond the original patient specimen and to investigate non-septic conditions, we analyzed five pairs of matching diseased and healthy (D/H) human tissue for the presence of eubacterial DNA. As anticipated by our previous findings[5] and those of others[18], using Bioanalyzer imaging, we detected bacterial 16S rDNA products in all ten tested specimens. When specifically analyzed for the presence of *E. hormaechei* DNA, two specimens were positive, both diseased aortic tissues. None of the matching healthy tissues tested positive for the presence of *Enterobacter* spp. DNA. Surprisingly, quantitation of *E. hormaechei* genomes in the diseased tissue using Q-PCR revealed the presence of 15 and 184 *E. hormaechei* genomes per 100 ng of total DNA isolated from the two positive samples, which equaled approx. 2×10^5 and 2.8×10^6 organisms per gram of tissue [20 mg tissue yielded ~30 μg total DNA using DNAEasy genomic DNA purification system (Qiagen)]. In addition, our *E. hormaechei* isolate harbors a plasmid and our data regarding resistance to ampicillin, cefazolin and erythromycin fall in line with published reports of antibiotic resistances that can be associated with *Enterobacter* spp. plasmids[24, 25]. *E. cloacae* have been identified in outbreaks, usually involving strains that overproduce beta-lactamase, including a nosocomial outbreak in a cardiothoracic intensive care unit[26]. It would be interesting to test serum samples for anti-*E. hormaechei* antibody titers; however, healthy tissue segments could only be obtained post-mortem and therefore serum was not available.

Persistence in Phagocytes

Most interestingly, *E. hormaechei* was the only species to be isolated from the atheromatous tissue using the methods described in this paper; therefore, we speculated that bacteria may be transported to sites of vascular injury after internalization by phagocytes. To address our finding, we hypothesized that *E. hormaechei* may be the only clinical isolate in the specimen due to the ability to persist intracellularly in monocytes. Although in other studies *Enterobacter* spp. have been shown to be invasive in epithelial cells[27], their persistence in macrophages[28] can be especially aggravating for an existing inflammation as phagocyte recruitment to the site would actually exacerbate the inflammation by delivering additional pathogens.

Applying the model of systemic dissemination via phagocytic cells, we tested the clinical isolate for adhesion and invasion of intestinal, phagocytic and endothelial cells, representing each consecutive step of the proposed model of dissemination. The isolate displayed invasion and persistence in monocytic THP-1 cells for up to 48 hours and high levels of attachment to intestinal epithelial and to endothelial cells Interestingly, the persistence of the clinical isolate was an order of magnitude higher than the persistence of the type strain *E. hormaechei* ATCC 49162, indicating higher ability for systemic dissemination. This is the first demonstration of increased intracellular persistence of clinical *Enterobacter* spp. isolate relative to the "domesticated"-type culture collection strain. In addition to the expected inter-strain differences, attenuation of laboratory strains is a previously observed phe-

Advance Publication
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nomenon. During sequential *in vitro* passage, laboratory reference strains might differentiate from non-passaged clinical isolates[29]. Most likely, the condition of the patient allowed the bacteria to bypass the intestinal tissue barrier and to leak in the circulation, accessing inflamed vascular sites directly or via phagocytic cell types. *Enterobacter* spp. are among the organisms previously isolated from infective endocarditis vegetation[30]. Although the isolate does not invade endothelial cells, invasion is not a prerequisite for pathogenicity. Enterohemorrhagic *Escherichia coli* (EHEC) strains associated with severe disease, such as the LEE locus career strain O157:H7, attach to the epithelial cell surface and also do not invade[31]. The effect of our *Enterobacter hormaechei* isolate on vascular cell types will be the subject of further exploration.

Only one enteric species was cultivated from this specimen, which suggests that only a subset of enteric species may be able to reach and survive at sites of vascular inflammation following systemic dissemination. This falls in line with the observation that the persistence of bacteria in macrophages varies from strain to strain[28]. Thus, the data presented here fit a model of systemic infection with an intermediate step where bacterial strains, once in the circulation, are internalized by phagocytic cells, at which stage selected species avoid immediate killing and spread and colonize distant sites. Bacterial internalization may also account for the resistance to antibiotic treatment observed in large randomized placebo-controlled clinical trials for the secondary prevention of cardiovascular events[32]. To address the study limitations, the true causative role of this species in vascular pathologies using *in vitro* and *in vivo* models must be further investigated.

Conclusion

This is the first description of *Enterobacter* spp. isolated from atheromatous tissue. In order to fulfill the "biological plausibility" of a role for bacterial infection in the pathogenesis of vascular walls, we describe here the isolation of the organism from atheroma, its characterization, and investigations on the interaction of this strain with the host cell types according to our mechanistic model of infectious agents' involvement with atherosclerosis. The isolate was able to survive in phagocytic cells for a period of time, potentially allowing for transmission to distant sites. In addition to being the only bacterial species isolated from this patient, it was detected in high numbers in additional aortic atheromatous specimens, but not in matched healthy controls. Taken together, the data suggest the possible association of *E. hormaechei* with diseased arterial (femoral or aortic) tissues. These findings warrant further studies of the interaction of this clinical isolate with vascular host tissues, and of the concept that bacterial persistence in phagocytic cells likely contributes to systemic dissemination.

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