P. aeruginosa Infection Increases Morbidity in Experimental Cholesteatomas

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Abstract

Objectives—Clinicians have long noted that infected cholesteatomas are more aggressive than uninfected ones without data to support these observations. The purpose of this study is to determine the etiological role of biofilm forming *P. aeruginosa* (PA) and the virulence factor, type IV pili, in the pathogenesis of experimental cholesteatomas.

Design—We evaluated three different PA strains and one *E. coli* strain in cholesteatoma progression: PA14, a well characterized wound isolate, OPPA8, an otopathogenic strain from a human cholesteatoma, OPPA8-NP, an isogenic type IV pili deletion mutant and DH5α, an *E. coli* strain.

Methods—Cholesteatomas were induced in gerbils. We inoculated the right ear with bacteria and the left with vehicle. After 6 weeks their cholesteatomas were evaluated by micro-CT scanning. Cholesteatoma size and bone resorption were analyzed digitally.

Results—Results demonstrate that PA infection increases cholesteatoma size when compared to uninfected controls: OPPA8 a showed an 8.9 fold increase, PA14 a 2.6 fold increase, OPPA8-NP a 1.9 fold increase while DH5α was not increased over controls. Additionally, infected bullae showed 10 to 50% more cholesteatoma-induced bone resorption.

Conclusions—In this model, PA infected cholesteatomas enlarge more rapidly and are more destructive than un-infected controls. OPPA8, the strain from a human cholesteatoma, showed the greatest enlargement and bone destruction. Additionally, we demonstrate that type IV pili is a virulence factor in this model since the non-piliated isogenic mutant, OPPA8-NP, was significantly less aggressive than the wild-type OPPA8 indicating that Type IV pili may be a virulence factor in this disease.

Keywords

Cholesteatoma; *P. aeruginosa*; micro-CT; gerbil; bone resorption

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INTRODUCTION

Cholesteatomas are cyst-like epithelial inclusions that develop in the middle ear and mastoid, usually as a consequence of chronic otitis media (COM). While the exact incidence of aural cholesteatoma is unknown, in well documented populations the prevalence of COM is about 1% and approximately 40% of those have cholesteatomas[1]. The incidence of COM varies widely in population groups. In the US, there are approximately 4000 procedures done for cholesteatomas each year at a cost of approximately 80 million dollars per year.

Aggressive cholesteatomas may also invade intracranially leading to meningitis and brain abscess. In a large reported case series 78% of subjects who had complications secondary to COM were found to have cholesteatomas[2]. Unlike uncomplicated otitis media, cholesteatomas cannot be eradicated by host defenses or pharmacological means. Since the lining wall of a cholesteatoma is keratinizing epithelium, keratin debris accumulates relentlessly within the lesion leading to gradual enlargement with characteristic destruction of surrounding bone. The only effective intervention is tympanomastoid surgery and removal of the lesion. Many cholesteatomas become chronically infected which tend to enlarge more rapidly and are observed by clinicians to be more destructive locally[3].

Most cholesteatomas develop by ingrowth of keratinizing epithelium into the middle ear rapidly contacting and eroding adjacent structures. Nearly all acquired cholesteatomas become chronically infected, most commonly with *P. aeruginosa* (PA)[4–6]. Brook and colleagues found that 85% of cholesteatomas contained bacteria[4]. Fulghum & Chole[6] found 93% of spontaneously occurring cholesteatomas in gerbils had bacteria. Cholesteatomas are characterized by increased adherence of bacteria to entrapped keratin and keratinocyte proliferation resulting in a 'matrix' of desquamated keratinocytes which forms an expanding mass. As chronically infected cholesteatomas enlarge, osteoclasts are recruited and activated leading to erosion of adjacent bone leading to ossicular destruction and hearing loss. Chronically infected cholesteatomas are more aggressive, recalcitrant to surgical and medical treatment and often recurrent[7–9]. We have previously demonstrated the presence of bacterial biofilms within cholesteatomas[9] and shown that PA isolated from human cholesteatomas form biofilms in vitro[10].

Mongolian gerbils, *Meriones unguiculatus*, are the only non-human animal known to develop spontaneous cholesteatomas[11]. These spontaneous cholesteatomas develop slowly and are more prevalent with increasing age[12]. An experimental model of induced cholesteatomas showed similar characteristics to spontaneous cholesteatomas[11]. In gerbils, the cholesteatoma develops within the ear canal and as it enlarges, displacing the tympanic membrane medially into the bulla where it erodes into the surrounding bony structures, including the external ear canal, ossicles, cochlea and bulla walls (Fig. 1).

In these studies, we used a modified protocol to inoculate the ear canals with PA prior to surgical ligation of the canal to determine the role of these bacteria in the etiology of cholesteatoma development. We evaluated three different PA strains: PA14, a well characterized and fully sequenced wound isolate[13], OPPA8, an otopathogenic strain isolated from a human cholesteatoma[10] and OPPA8-NP, an isogenic type IV pili deletion mutant generated in our laboratory (see methods) and DH5α, an *E. coli* laboratory strain. (See methods).

MATERIALS AND METHODS

DESIGN AND RATIONALE

Bilateral cholesteatomas were induced in 27 gerbils. One ear was infected with the indicated bacteria and the other with sterile vehicle only. At six weeks microCT scans were performed and the extent of the cholesteatoma and bone changes were measured. Four strains of bacteria were chosen:

PA14 A laboratory strain of *P. aeruginosa* was obtained from Alice Prince (Columbia University, NY). This strain, UCBPP-PA14, was originally isolated from a wound by M. Schroth at the University of California, Berkeley.

OPPA8 A strain of otopathogenic *P. aeruginosa* isolated from a patient with an infected cholesteatoma. We have characterized this strain previously. [10] This strain is a competent biofilm former and we hypothesized that it would be the most virulent in this experimental model.

DH5α A standard laboratory strain of *E. coli*. Used as a comparison strain of a gram negative bacteria that forms LPS but has not been shown to be virulent in cholesteatomas.

OPPA8-NP A strain devoid of type IV pili derived from OPPA8 This strain lacks type IV pili which are required for colonization of host tissues and for twitching motility. Adherence is thought to be the first step in the formation of microcolonies and hence biofilms. The strain is identical to OPPA8 except for the lack of type IV pili. Our hypothesis is that type IV pili are a virulence factor in chronically infected cholesteatomas.

OPPA8-NP Construction

Primers were designed to 5' and 3' flanking regions of the *pilA* gene containing restriction sites at the ends (5'forward- ccagcggccgccggttttctccgcaggcaagtcg, 5'reverse- ccaggatccccgagcgaatgccgctaatagg, 3'forward- ccaggatccctccattgatatatccaggcctaacgc, 3'reverse- ccagtcgacccacaacggaactactcgatgctgg). PCR products amplified from genomic OPPA8 DNA were digested with the corresponding enzymes and ligated at the SalI sites and simultaneously ligated into a suicide plasmid, pJB4648, at the NotI and BamHI sites. The suicide plasmid contains genes for gentamicin resistance (GentR) and sucrose sensitivity (SacB). The resulting plasmid containing the fragments was transformed into OPPA8 by electroporation. Plasmid integrants were selected by plating on LB with gentamicin (20μg/mL). Colonies were then plated on LB with 4% sucrose to select for loop-outs of the *pilA* gene. True deletion mutants were confirmed by PCR of sucrose resistant colonies.

Experimental cholesteatoma

Four to six week old Mongolian gerbils, *Meriones unguiculatus*, were obtained from Charles River Laboratories (Wilmington, MA). Use of these vertebrate animals was approved by the Washington University Animal Studies Committee. Cholesteatoma was induced by surgical ligation of the external auditory canal as described by McGinn, *et. al.*[11] Briefly, the gerbils were anesthetized with a mixture of ketamine HCl (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, 0.08mg/g)/xylazine HCl (supplied by the Department of Comparative Medicine, Washington University, St. Louis, MO, 0.02mg/g) injected intraperitoneally. Bupivicaine HCl (Marcaine, Hospira, Inc, Lake Forest, IL, 0.1μg/g) was also given for pain relief. A small postauricular incision was made, 20μL sterile PBS was inserted into the left external auditory canal, which was ligated with 4-0 silk suture around the cartilaginous canal. This process was repeated using 20μL of a *P. aeruginosa* (PA) strain diluted in PBS and inserted into the right auditory canal. For preliminary experiments, PA strain OPPA8 was used at

10^4–10^8 cfu/mL. These animals were sacrificed 1 and 2 weeks post operatively for microbiological, histological, and preliminary micro-CT evaluations. For animals that underwent controlled micro-CT evaluation, four groups of six animals were infected with strains PA14, OPPA8, OPPA8-NP, or DH5α at 10^7 cfu/mL (2×10^5 cfu per ear inoculation), and one group of three animals were used as untreated controls. One animal from the PA14, OPPA8-NP, and control groups and two animals from the OPPA8 group and one from the OPPA8-NP group died before the end of the experiment and were excluded from the analysis. This study was approved by the Washington University Animal Studies Committee.

Micro-computed tomography and histomorphometry

Micro-computed tomography (micro-CT) was used to assess the level of bone erosion and reactive bone formation present in the gerbil ear. Six weeks after surgery, animals were given sodium pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA, 0.5mg/g) to anesthetize them before perfusing with 2% paraformaldehyde, 2.5% glutaraldehyde in a 0.1M phosphate buffer (pH 7.4). The intact head from each animal was oriented in a precise axial orientation within the 36 mm diameter plastic cylinder. The cylinder with the properly oriented head was filled with agarose for stabilization. The micro CT scanning was performed on a Scanco μCT 40 (Scanco Medical AG, Basserdorf, Switzerland) with point-spread function of 0.036 mm over a period of 2–3 hours per specimen. The scanning parameters in the standard mode consisted of 70 kVp tube potential, 114 mA tube current, 200 ms integration time. The resulting reconstructed volume had 36 μm isotropic voxels. For all analyses, the raw CT image was modified in Adobe Photoshop® (Adobe Systems Inc., San Jose, CA) in a standard manner; levels converted from 0–255 to a contrast range of 0–150 to better differentiate gas from tissue and tissue from bone. The grayscale images are saved as *.tif files for analysis with ImageJ® (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2006). For histomorphometry, axial microCT images were selected from a level that intersects both external auditory canals and includes the mid-portion of the cochlea. All axial images were coded and blinded for image analysis.

For cholesteatoma size, radiolucent areas, presumably trapped air or gas, were delineated and saved; radio-opaque areas, presumably cholesteatoma, were separately delineated and saved (Fig. 2 and 3.) The separately captured images were converted to black and white images and saved as *.tif files. Using NIH ImageJ®, the area of the gas and the cholesteatoma were measured (calibrated directly from CT image). The proportion of cholesteatoma (radio-opaque area) was expressed as a percent of total: cholesteatoma/(gas + cholesteatoma). Results were recorded as percent of tissue within total bulla area within each axial section.

For bone erosion measurements, a standard rectangular box was placed over the lateral bulla between the buttresses. (Fig. 3) The image was captured and saved as a *.tif file. Using NIH ImageJ®, the length of the bone was subtracted from the total distance across that area of the bulla and was expressed in mm. The external auditory canal is in this region and is seen as a deficient area of bone of the lateral bulla (rectangle on the right). Erosion of this area is typically seen in gerbil cholesteatomas and is evident by the larger defect on the right side compared to the right (Fig. 3). Results were recorded as length of bone divided by total length between buttresses. All parameters were measured in three reproducible frames.

The outlines were then converted to bitmap images and the number of pixels was calculated using ImageJ software (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2006). Statistical significance across was evaluated using standard parametric statistics (*i.e.* paired t-test). An α level of 0.05 was

established for significance. All statistical calculations were performed with SigmaStat software (version 3.5, Systat Software, Richmond, CA).

The histomorphometric measurements were highly reproducible. When measurements of cholesteatoma size were analyzed by two separate, blinded observers, the ratio of infected to uninfected cholesteatoma size for OPPA8, OPPA8-NP and PA14 were 9.3, 2.3 and 3.0 from one investigator (D.L.,) and 8.9, 1.9 and 2.6 (R.C).

Microbiology

Swabs were taken from the ears of a subset of the gerbils in the preliminary study at the time of sacrifice. The swabs were streaked onto P. Isolation Agar (PIA, Remel, Lenexa, KS) plates to obtain PA colonies for further PCR identification. The swabs were then sent to the Clinical Microbiology Laboratory of Barnes Jewish Hospital, St. Louis, MO to identify other species present. Four colonies from each swab were selected for colony PCR to recover OPPA8. Colonies were re-suspended in 25μL dH$_2$O and boiled for 10 minutes. The PCR contained 1X buffer, 0.2mM dNTPs, 1.5mM MgCl$_2$, 2μL boiled colony DNA, 5U Taq polymerase, and 0.5μM of each primer, (forward-ccggtgtgactggctctatt, reverse-tgccatcctcctgctatttc). The reaction was carried out at 94°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute,

RESULTS

To determine the etiologic role of *P. aeruginosa* in cholesteatoma development, we inoculated bacteria (right ears) or PBS (left ears) prior to surgical ligation. Animals were sacrificed after 6 weeks, fixed in mixed aldehydes and imaged by micro-CT. For each animal, three reproducible sections were quantitatively analyzed and representative axial images of experimental gerbil cholesteatomas are shown in Figure 4. For cholesteatoma size, results are shown as ratios between experimental and control (mean ± standard deviation). For bone erosion, results are shown as percent eroded surface, bacterial minus control (mean ± standard deviation). The p values above each group represent paired t-tests comparing control and bacteria. (Fig.5) For these experiments, we consistently inoculated the left ear with PBS and the right ear with bacteria, using each animal as its own control. Gerbils were coded at the time of surgery and images were blinded to the individual doing the analysis (RN).

Results demonstrate that inoculation with PA into the ear canal prior to ligation dramatically increases both the size of the cholesteatoma and the amount of bone resorption when compared to the un-infected contralateral ear. OPPA8 showed 8.9-fold, OPPA8-NP 1.9-fold and PA14 2.6-fold increased size compared to the un-infected contralateral ear (Fig. 5). OPPA8, the cholesteatoma-derived PA strain, induced significantly larger cholesteatomas than both the non-piliated strain, OPPA8-NP and the laboratory wound-derived PA strain, PA14 (p<0.05). Importantly, the Escherichia E. coli strain DH5α, did not induce increased expansion of the cholesteatomas formation. (Fig. 5)

Cholesteatoma-induced bone erosion for OPPA8, OPPA8-NP and PA14 were increased 156%, 120% and 120% compared to un-infected controls, respectively and the DH5α E. coli strain did not induce bone erosion (Fig. 5). Interestingly, the cholesteatoma-derived PA strain demonstrated significantly more erosion than the laboratory strain PA14 (Fig. 5 p=0.025).

We verified the presence of the cholesteatoma by gross dissection of the bulla. Additionally, appropriate *P. aeruginosa* strains were recovered in the cholesteatomas by PCR (data not shown).

DISCUSSION

P. aeruginosa is the most common pathogen associated with nosocomial pneumonia and burn infections [14] and continues to be a leading cause of morbidity and mortality in cystic fibrosis patients [15]. It is also a common cause of corneal keratitis[16], urinary tract infections[17], and external otitis[18]. Our laboratory has shown that PA is also an important pathogen associated with aural cholesteatomas[10, 19]. In fact, it is the most common gram negative organism isolated from human cholesteatomas, accounting for 21% to 51% of the organisms cultured[6, 10]. PA is also the most common gram negative organism cultured from experimental cholesteatomas in gerbils[6].

One of the hallmarks of aural cholesteatoma is chronic and recurrent infection, which is highly resistant to eradication, often despite multiple courses of antibiotics. Once a cholesteatoma is infected, chronic otorrhea usually occurs. Although suppression can occur with topical and systemic antibiotics, recurrence is common. The ability of PA to form biofilms may explain many of these clinical observations. Previous studies in our laboratory have demonstrated histological and ultrastructural evidence that bacterial biofilms are common in human and experimentally induced gerbilline cholesteatomas[9]. We have also demonstrated that a majority of PA isolates from cholesteatoma (otopathogenic PA or OPPA) have increased adhesion to keratinocytes and increased biofilm formation compared to a common environmental strain (PA01)[10].

In our previous study of ligation induced cholesteatomas in gerbils, the rate of cholesteatoma formation is highly variable, with only 40 to 70% of ligated ears developing cholesteatomas and these generally did not expand beyond the confines of the tympanic membrane and external ear until *4–6 months* after ligation[11, 12]. In this current model, 100% of ligated ear canals inoculated with Pseudomonas aeruginosa demonstrated radiographic evidence of cholesteatoma development after *only six weeks*, suggesting that infection with PA results in highly aggressive cholesteatomas. Quantitative histomorphometric measurements demonstrated increased cholesteatoma size and bone resorption in response to PA infection. The largest and most destructive cholesteatoma was induced by OPPA8, a cholesteatoma-derived PA strain. Interestingly, cholesteatoma size and bone erosion was attenuated in a non-piliated OPPA8 strain, suggesting that this effect is dependent on the presence of type IV pili (TFP), which is considered a virulence factor because of it's role in twitching motility and surface adhesin.. In cystic fibrosis, TFP have been shown to be an important mediator of adhesion to pulmonary epithelium[20], virulence in animal models[21], and in immune recognition, with their hypervariable regions involved in antigen formation[22, 23]. The decreased virulence that we observed using the non-piliated mutants may be due to diminished ability to form biofilms since adherence of these bacteria to keratinocytes is likely impaired.

These results demonstrate an etiologic role for PA in the development of larger, more aggressive cholesteatomas. Since the *E. coli* strain, DH5αa did not accelerate cholesteatoma expansion, our results are suggestive of a specific interaction of *Pseudomonas* species within the ear canal resulting in dramatic enlargement and aggressiveness of the cholesteatomas. The complete lack of response to *E. coli* further suggests that simple presence of bacteria is insufficient to account for the enhanced aggressiveness of these cholesteatomas. Additionally, there was significant attenuation in the non-piliated strain, suggesting a role for type IV pili in the pathogenesis of PA-induced cholesteatomas. Strikingly, medial expansion of cholesteatomas occurred in every PA-infected ear canal much more rapidly than previously reported un-infected cholesteatomas (6 weeks compared to 6 months).

CONCLUSION

When infected with PA, experimental cholesteatomas enlarge more rapidly and are more destructive than un-infected cholesteatomas. An otopathogenic strain, OPPA8, isolated from a human cholesteatoma, was the most aggressive. The virulence of this strain was diminished by deletion of its principal adherence structure, type IV pili. Comparison with laboratory strains, PA14 and *E. coli* (DH5α) likewise showed less virulence.

Acknowledgments

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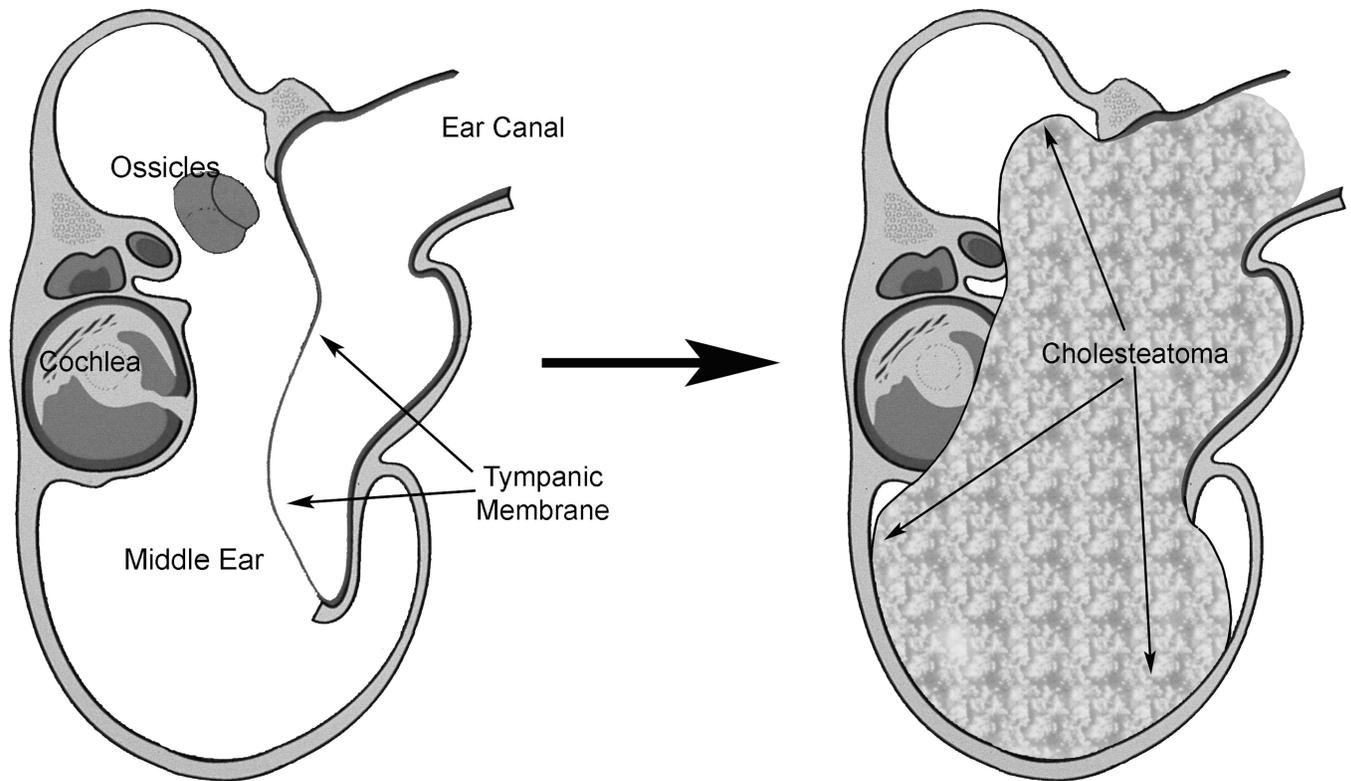
Figure 1.
Diagram of cholesteatoma development in the gerbil model. After surgical ligation of the ear canal, keratin debris fills the canal. As it expands, it erodes into adjacent bone, leading to hearing loss.
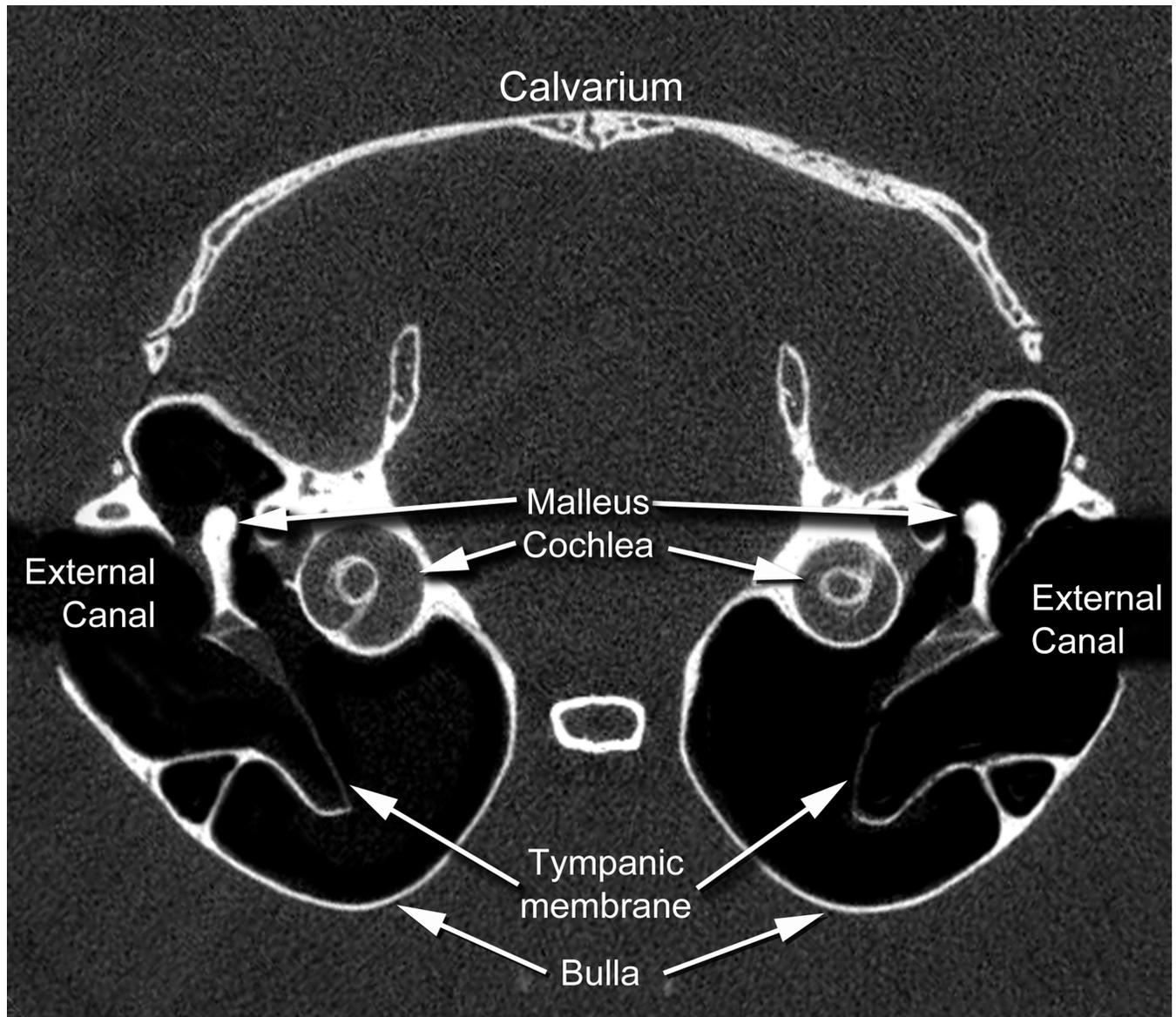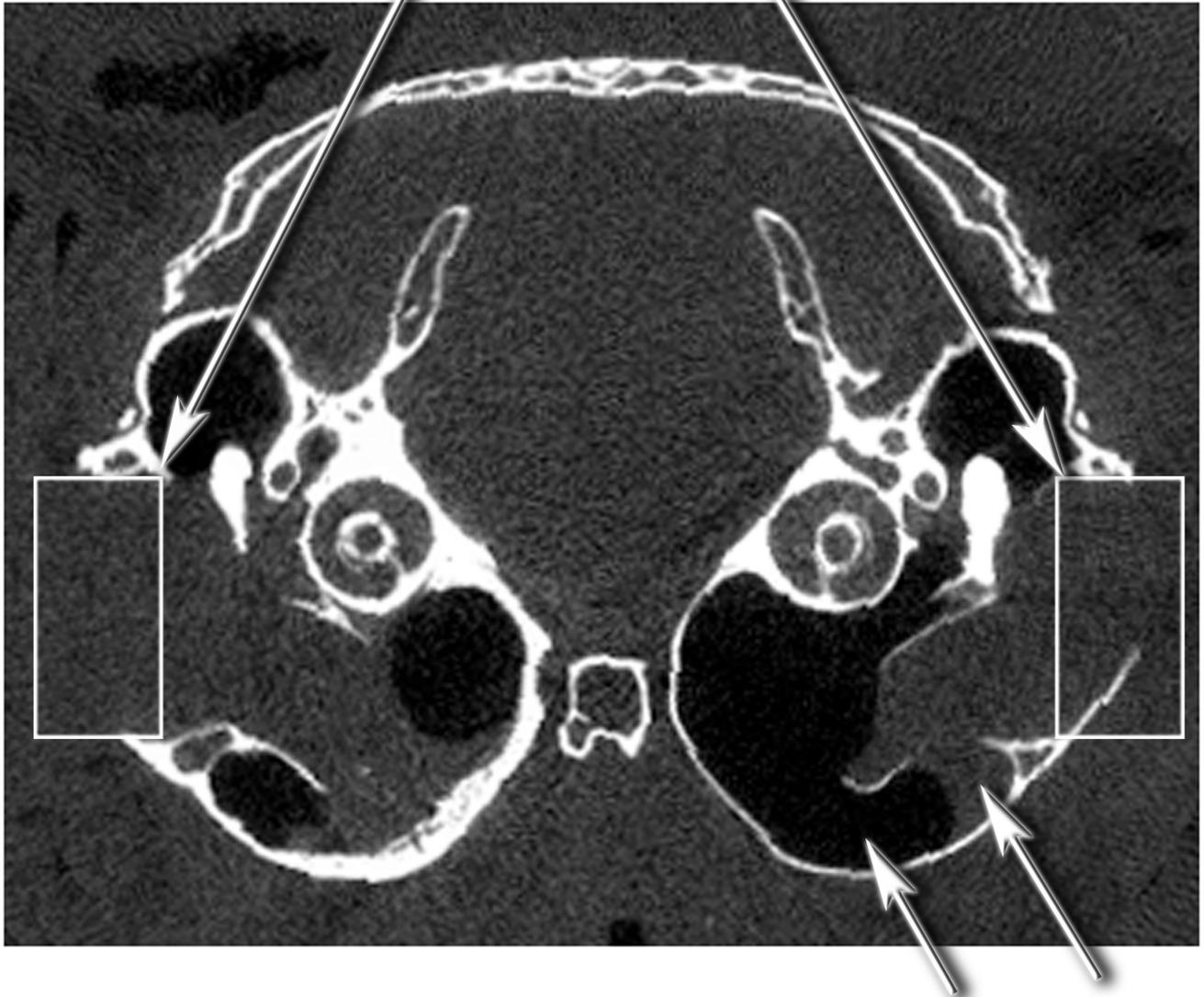
Figure 2.
An axial microCT scan of a normal gerbil head demonstrates the position of the bullae, ear canals, calvarium, malleus and cochlea.

Figure 3.
An axial microCT scan of a gerbil head with induced cholesteatoma demonstrating morphometric measures. The area of bone resorption was determined by the length of remaining bone in a standardized box. The area occupied by the cholesteatoma was determined by the area of soft tissue (radio-opaque) divided by the area of soft tissue plus area of gas (radiolucent)
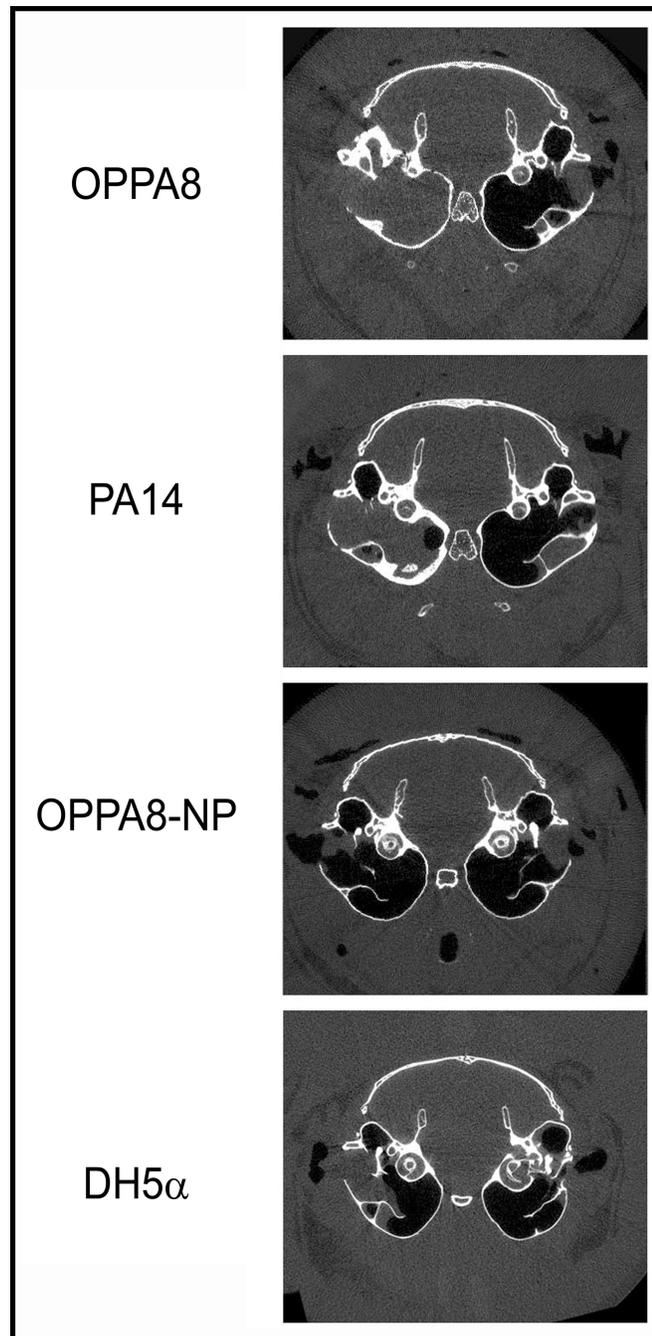
Figure 4.
Representative axial microCT scans of the experimental animals. Indicated bacteria were inoculated into the induced cholesteatomas on the right side (left side of image) and vehicle only was introduced into the left side (right side of image.) Note that OPPA8 and PA14 scans show increased cholesteatoma size and bony change compared to the non-piliated OPPA8 and the E. coli strain DH5α.
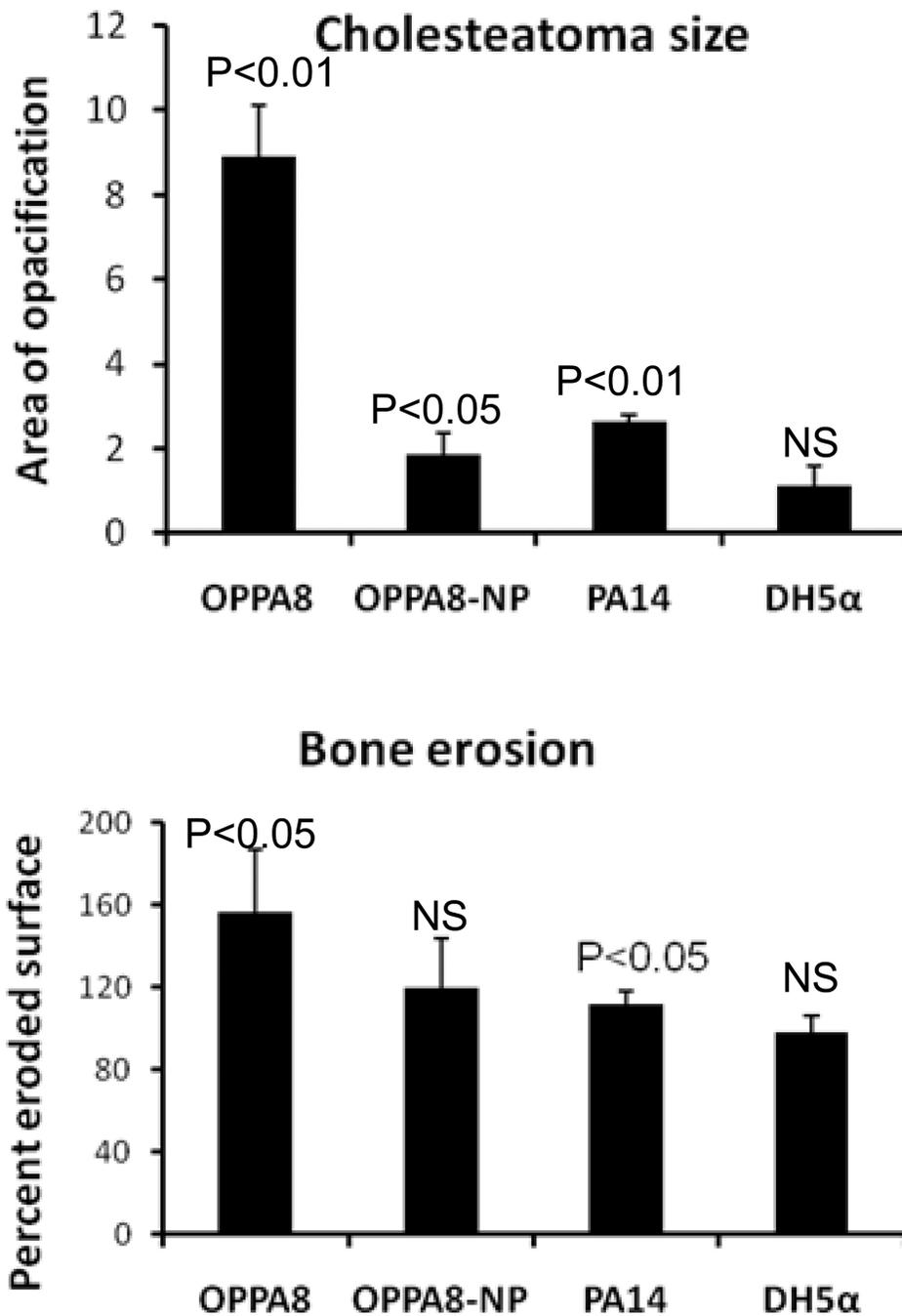
Figure 5.
Upper panel, *P. aeruginosa* infection increases the size and aggressiveness of cholesteatomas. The Otopathogenic strain (OPPA8) causes the most growth. PA14 and OPPA8-NP cause modest growth compared to controls but significantly less than OPPA8. DH5α (E. coli) causes no more enlargement than the control (vehicle only). Lower panel, Significant bone erosion was seen with infection with OPPA8 and PA14 but not with OPPA8-NP or DH5α.