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Bacterial biofilms in cholesteatomas of Mongolian gerbils

Angela Clabots

Bacterial Biofilms in Cholesteatomas of Mongolian Gerbils

by

Angela Clabots

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INTRODUCTION

Cholesteatomas are cyst-like growths occurring within the pneumatized portions of the temporal bone. They are normally caused by negative middle ear pressure due to Eustachian tube dysfunction, infection, or chronic middle ear effusion. Initially the tympanic membrane loses structural support and retracts to form a pocket. Since the pocket is part of the squamous epithelium of the tympanic membrane, keratin is continually being sloughed off. Eventually the pocket becomes filled with desquamated keratin and purulent material. The cholesteatoma tends to invade and destroy the middle ear system if it is not surgically removed in time. Both mechanical and biochemical factors play a role in the demineralization and decay of the auditory ossicles (Canalis & Lambert 2000). This study focused specifically on cholesteatomas due to their recurrent nature and location, which allows for bacterial growth plus easy dissection.

The Mongolian gerbil is found to have spontaneously occurring cholesteatomas, containing flora similar to that found in human cholesteatomas (Fulghum & Chole 1985). Studies of cholesteatomas induced by ligation in many different animals showed that gerbils produce the fastest keratin growth, which causes tympanic membrane displacement, and bony erosion (McGinn, Chole & Henry 1984). The similarities between human and Mongolian gerbil cholesteatomas suggest that the gerbil is a good model to use for research on cholesteatomas. One aim of the present study addressed was to assess whether Mongolian gerbils still form cholesteatomas under current improved cleanliness standards typical of modern animal housing facilities. The results of this study are compared to those obtained by Fulghum and Chole in 1985 and Fortuny et al. in 1993.

A group of gerbils were administered antibiotic treatment to see if the treatment changed the growth of the cholesteatoma or bacterial formation within the matrix.

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Another focus of this study was biofilm formation, a very recent topic of interest due to the destructive nature of biofilms within the medical arena. Biofilms are bacterial colonies that flourish in warm, moist locations. They are organized communities and most are found containing water channels allowing the flow of nutrients and wastes throughout the matrix. Although biofilms are an everyday part of our lives, used in filtering industrial water, bioremediating hazardous waste sites, and protecting soil and groundwater from contamination, they are extremely damaging to equipment and costly to hospital patients (CBE 1999). These colonies of bacteria within a biofilm are extremely resistant to antibiotics and disinfecting agents. The resistance may be due to the selectively permeable biofilm layer or the dormant cells deep inside the colony where the antibiotics never penetrate. Stewart and Costerton 2001 suggest the possibility that aminoglycosides are ineffective if the bacteria are anaerobic. The most helpful way to identify biofilms is with a laser scanning confocal microscope. However the architecture of each biofilm is substrate dependent, making it difficult to define a generic size and structure for biofilms (Sutherland 2001). Biofilms are present in every puddle outside, on our teeth, and even on hospital prosthesis. One study found that oral biofilms became denser and thicker when supplemented with sucrose (Sutherland 2001). Biofilms can attach to any surface and tend to grow when the conditions are good, but close themselves off when being threatened by antibacterial agents or other harsh conditions. The bacterial biofilm Pseudomonas aeruginosa was found in the sputum of cystic fibrosis patients who fight recurrent pneumonia (Costerton 2001). In 1993-1994, 100 albuterol inhaler users died due to Pseudomonas aeruginosa present in their inhalers (Costerton & Stewart 2001). Medical implants are known to cause slow-burning infections, and it is thought that the underlying cause of about 65% of medical infections is due to biofilms (Boyd 2000). Biofilms have also been found in chronic tonsillitis and sinusitis (Ozeki

1997). The middle ear is the ideal environment for biofilm formation, and biofilms are indeed present and quite likely a causative factor in chronic otitis media with effusion according to Post (2001). The current study hypothesized that biofilms would be present within the induced cholesteatomas.

MATERIALS AND METHODS

Animals

Thirty-four male Mongolian gerbils were used in this study. Data were collected from a total of forty-eight bullae from the non-antibiotic group, and twenty bullae from the antibiotic group. All the animals were from Charles River Laboratories. Animals were housed on a 12:12 h light:dark cycle with food and water available ad libitum. Four animals each were sacrificed at 12 and 16 weeks post-ligation (+- 6days). Eight of the gerbils received Trimethoprim-sulfamethoxazole at approximately 30 mg/kg BW/day from their water beginning one week post-ligation. Half of the antibiotic group was sacrificed at 24 weeks, and the rest at 30 weeks post-ligation. The remaining gerbils were sacrificed at 30 weeks post-ligation. Any unusual behavior was noted prior to sacrifice. The Animal Studies Committee at Washington University approved the care and use of the animals used in this study.

Ligation surgery

Initially the animals were anesthetized with Ketamine/Xylazine and the hair around the anterior-ventral pinna was removed by plucking. That area was swabbed with antiseptic and a 0.5 cm incision was made just anterior to the pinna. A suture was looped around the ear canal under the skin with sterile curved forceps and pulled the ear canal closed. The suture ends were tied and left under the incision, which closed within 24 hours. The incision was not sewn shut because littermates actually gnaw at any sutures therefore causing complications.

Tissue Harvest

At a specified date post-ligation, the animals were sacrificed with an overdose of pentobarbital and perfused transcardially with a solution of 2% paraformaldehyde and 2% glutaraldehyde. The heads were removed and the external auditory canals were inspected to make sure the ligations were still intact. The bullae were then opened and the contents examined to assess the developmental stage of the cholesteatoma. Many of the bullae were prepared for sectioning by removing both ends of the bullae allowing for complete immersion in the phosphate buffer fixative of 4% paraformaldehyde overnight.

Histology

The samples were then submersed in .35M tetra-Na EDTA for 4-10 days to decalcify the bullae. The bullae were rinsed with 0.1M PBS. Then the samples were dehydrated using 70%, 80%, 90%, and 100% acetone for 30 minutes each. Infiltration was done using ratios 1:2, 1:1, and 2:1 Epon to Acetone for 4 or more hours. Embedding was done in 100% Epon and BDMA (1 drop BDMA per ml solution) to make the specimen hard enough for knife sectioning. Then the cholesteatomas were cut into semi-thin sections (1.0um) and placed on a glass slide. The sections were counterstained with 1% toluidine blue and coverslipped with permount. Adjacent sections were prepared for gram-staining using Protocol Gram Stain Set from Fisher Diagnostics. The slides were viewed with an Olympus BH2-RFCA microscope and pictures were taken using a Sony DKC-5000.

Bacteriology

Portions of cholesteatoma matrix were placed in sterile saline and sent for bacteriology through the Division of Comparative Medicine at Washington University School of Medicine. The first several were sampled for aerobic and anaerobic bacteria, however no anaerobic bacteria

were present. Therefore rest of the cholesteatoma matrices were only sampled for aerobic bacteria. Comparisons were also made between the bacteria present at different cholesteatoma stages, and whether these bacteria were present in the two previous studies.

One group of animals was administered the antibiotic Trimethoprim-sulfamethoxazole in their water. Through oral administration, this antibiotic is distributed to middle ear fluid and is known to be effective on bacteria found in the middle ear, such as Streptococcus pneumoniae and Escherichia coli. The antibiotic group was compared to the others and any differences in the cholesteatoma or bacterial isolates present was noted.

Analysis

A subjective assessment was performed to identify the presence and stage of cholesteatomas in the gerbils. The cholesteatoma stages are defined by McGinn et al. 1984 as follows. A stage I gerbilline cholesteatoma is comprised of small keratin deposits on the outer surface of the tympanic membrane. Progression to Stage II is when the tympanic membrane bulges into the middle ear space, but does not contact any walls of the bulla or otic capsule. Stage III involves contact of the cholesteatoma with the cochlea. Stage IV is where the cholesteatoma fills the entire bullae. A Stage V cholesteatoma involves intracranial extension beyond the bullae. Comparisons were made between the different post-ligation dates, between ears, and between the control and antibiotic group.

The presence of biofilms was performed subjectively through an electron microscope, since that is currently the "gold standard" for identifying biofilms. Bacteriology was performed to identify which bacteria were present at different cholesteatoma stages, and to look for agreement with bacteria found in two previous studies. A comparison of bacterial flora was also done between the antibiotic group and the control group.

RESULTS

Animal appearance

Initially the plan was to sacrifice the animals 3 months post-ligation, and then every four weeks after that to obtain different stages of cholesteatomas, however this was not necessary since the first group already possessed the entire range of cholesteatoma stages. The ligation itself had no external effects on most of the gerbils. Three gerbils had noticeable head tilts prior to being sacrificed. The head tilts were due to the cholesteatoma either invading the vestibular system or the brain stem. Some gerbils also obtained large bumps behind their pinna due to the extensive growth of the cholesteatoma beyond the bony shell of the bullae. However, not all of the animals with stage 4 cholesteatomas had balance disturbances or lumps behind their pinnae. The antibiotics appeared to have no effect on the normal rate of development in the gerbils.

Histology

All stages of cholesteatomas were present and harvested, with a higher predominance of stages two and three (Figure 1). Some of the ligated ears did not contain cholesteatomas, probably due to the ligation not holding. Bacteria were seen as small colonies throughout the cholesteatoma matrix (Figure 2). The gram staining showed that some biofilm communities were predominantly one shape, while others were a mix of gram-positive and gram-negative species (Figure 3). A variety of stages were found at all post-ligation periods examined (Figure 4). At 24 weeks post-ligation, there appeared to be no difference in the cholesteatoma stages between the antibiotic group and the rest of the gerbils (Figure 5).

Bacteriology

Each cholesteatoma contained between three and five bacterial isolates, all of which were aerobic. The most common bacteria was Enterococcus faecalis (group D), which was present in

75% of the twenty samples sent for bacteriology. Other bacteria found in over half the samples are Proteus mirabilis, Staphylococcus aureus, and Escherichia coli (summarized in Figure 6).

At 24 weeks post-ligation, both ears of four gerbils (13-16) were sent for bacteriology. When comparing bacteria from right and left cholesteatomas, 12 organisms were present bilaterally, however six organisms were not found in the contralateral ear of the gerbil. A comparison of bacterial isolates present during each post-ligation period revealed no correlation between the strains of bacteria and the number of weeks post-ligation (Figure 7).

Bacteria in Different Stages

Multiple bacteria were present in each stage of the cholesteatoma (Figure 8). No relationship was found between the amount of bacterial isolates present and the stage of the cholesteatoma. The stage five cholesteatoma had only three isolates, whereas all of the stage two cholesteatomas had four or more bacteria present. Also, no relationship could be noted between cholesteatoma stage and strains of bacteria present.

Bacteria in different stages: Antibiotic Group

Four samples from the antibiotic group were sent for bacteriology. The findings from this group were similar to the non-antibiotic group. Both groups grew between three and five bacterial isolates in each cholesteatoma (refer back to Figure 6). The most common bacteria present were also Enterococcus faecalis (group D), Proteus mirabilis, Staphylococcus aureus, and Escherichia coli. Interestingly Enterococcus avium and Pseudomonas aeruginosa were found only in one animal, which was in the antibiotic group. This antibiotic however is not particularly effective against Pseudomonas aeruginosa.

DISCUSSION

Major findings

The stages of the cholesteatoma had no effect on the type or amount of organisms present. The cholesteatomas did contain some of the same bacteria as previous studies, but also some organisms were isolated that were not previously found. No anaerobic bacteria were present. These results suggest that the cleaner housing facilities had no impact on the types of bacteria the gerbils grew. Biofilms consisting of different shapes and sizes were identified in each cholesteatoma within the layers of keratin.

Potential problems

Those bullae found clear of any keratin build-up were not necessarily resistant to the growth. Most likely the ligation was not tight enough, creating incomplete closure of their external auditory canals. If the ligation did not hold, then keratin from the tympanic membrane could migrate out of the external auditory canal therefore avoiding a retraction pocket. One disadvantage to ligating the ear canal was the inability to view the cholesteatoma as it grows.

Another potential problem was the method of administering the antibiotic. Due to the antibiotic being put in the animal's water, it was not possible to regulate the amount of antibiotic each animal received. It was also unknown how long the antibiotic remained effective sitting in water. Plus the weight variation among the gerbils was huge, and would need to be accounted for by dosage in order to judge the effectiveness of antibiotic treatment. It is possible that a much larger dose of Sulfamethoxazole may have had an effect on the bacteria within cholesteatomas, however even if the antibiotic stopped the current infection, the biofilm would still be present and waiting for the right environmental conditions to begin growing again.

How results compare/contrast to prior findings

Comparing present bacteria findings with those of Fulghum and Chole 1985, there were four organisms found in both studies. Three of the bacterial isolates identified in the current study were also found previously by Fortuny et al. in 1993, but only one of them agreed with Fulghum and Chole's findings. The current study and Fortuny et al. 1993 did not find any anaerobic organisms. Fulghum and Chole 1985 did find anaerobes, which were also seen in human cholesteatomas by Brook in 1981. There were many bacteria previously found in both studies that were not present in this study, which may be explained by the sterile animal housing facilities currently used. However there were four new bacteria present in these cholesteatomas, which were not present in either of the previous studies. These new findings may represent the wide variety of bacteria present in Mongolian gerbil ear canals, however research by Fortuny et al in 1993 found no correlation between bacteria in control external ear canals and induced cholesteatomas in contralateral ears.

Based on literature, are these bacteria found typical biofilm formers?

One of the most commonly researched bacterial biofilms is Pseudomonas aeruginosa, which was present in only one animal in the current study. Many of the organisms present have not been identified as typical biofilm formers, and may not have been contained within a biofilm matrix.

Future research

By using Mongolian gerbils as a model for human cholesteatomas, there is the capability of inducing different strains of bacteria into the cholesteatomas to compare lab strains with potent disease-causing strains. There is also great potential for comparing the effects of new antibiotic treatments on biofilm formation within cholesteatomas.

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FIGURE LEGEND

Figure 1. All stages of cholesteatomas were examined in this study. There was a predominance of cholesteatoma stages two and three.

Figure 2. Low power photomicrograph of a region of cholesteatoma matrix containing several colonies of bacteria indicated by arrows. The bacteria typically colonize deep within the layers of desquamated keratin where they are presumed to be inaccessible to antibiotic treatment. Plastic section counterstained with toluidine blue.

Figure 3. High power photomicrograph of a bacterial colony within the choleateatoma matrix, gram-stained. Note both gram-positive (dark blue) and gram-negative (pink) profiles co-exist in the same colony.

Figure 4. A variety of cholesteatoma stages was seen at all post-ligation periods examined. The amount of time post-ligation did not affect the stage of the cholesteatoma. From 12 to 30 weeks post-ligation there was still an average distribution of stages 1-4 present.

Figure 5. Analysis of cholesteatoma stages present at 24 weeks post-ligation reveals no significant differences between the antibiotic and the untreated group. Both groups had (on the average) more stage 2-4 cholesteatomas than any other stage.

Figure 6. A comparison of organisms isolated from antibiotic and untreated animals revealed no significant differences between the bacteria found in each group. The most common isolates found in the untreated group, were also present in the antibiotic group. The percentages are not comparable due to the small sample size of the antibiotic group.

Figure 7. Analysis of isolated bacteria across post-ligation time showed that most cholesteatomas contained three or more isolates regardless of the sacrifice date. The bacteria found in each organism are indicated with the X. Some organisms appear much more often than

others. However, the period of time post-ligation does not appear to be related to the amount of

bacterial isolates present. Those bacteria isolated at 12 weeks were also found at 24 weeks post-

ligation.

Figure 8. Analysis of isolated bacteria across cholesteatoma stages showed various isolates

present at each stage of cholesteatoma growth. The number and type of bacteria present was

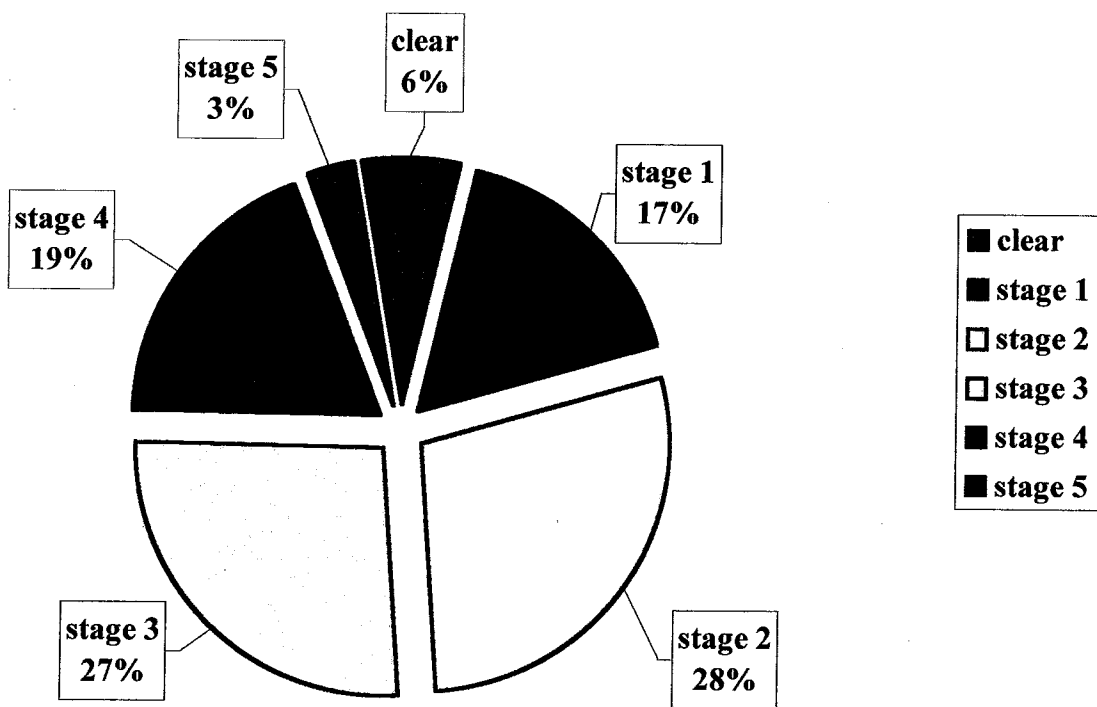unrelated to the stage of the cholesteatoma.
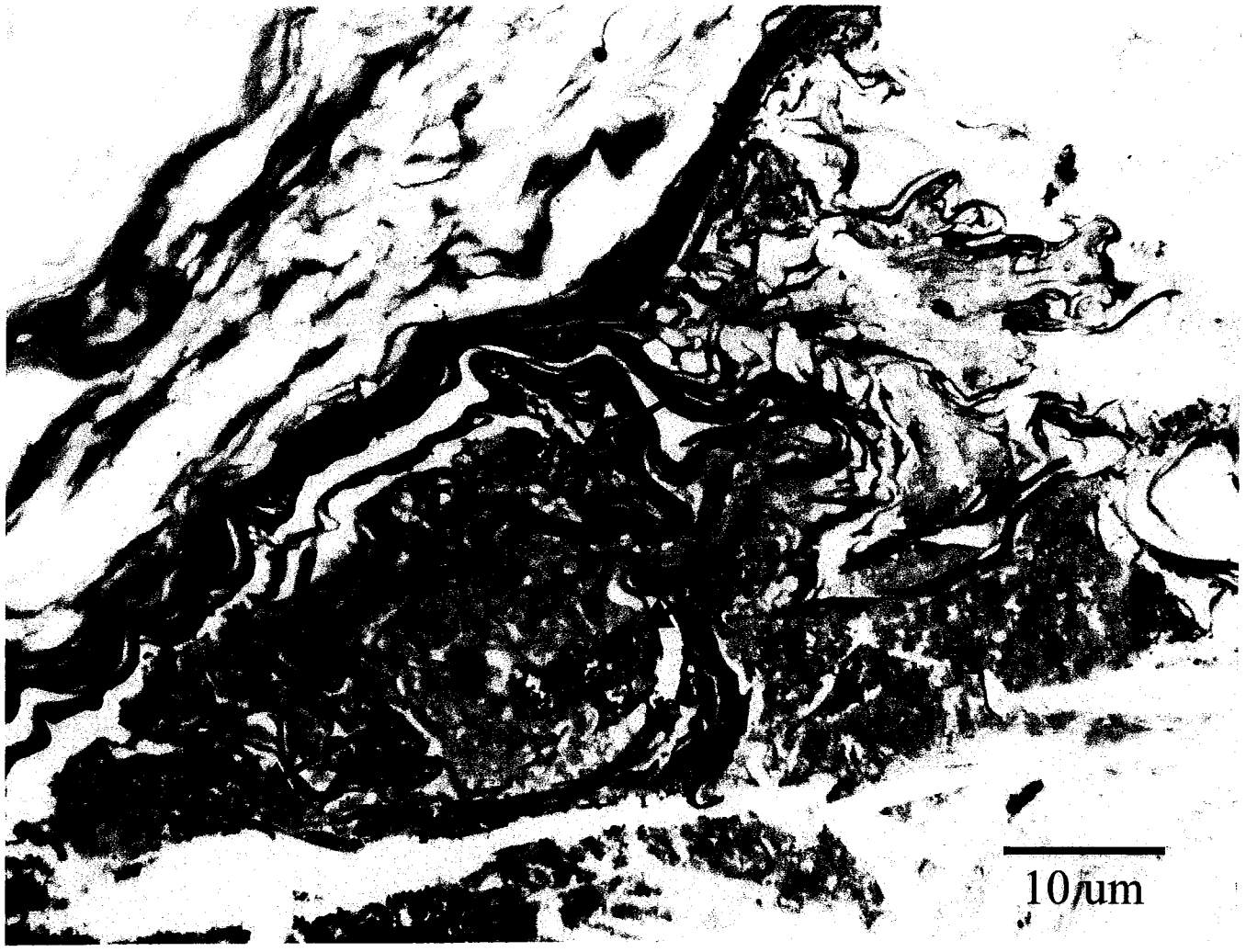
Cholesteatoma stages present

Figure 1.

10 µm
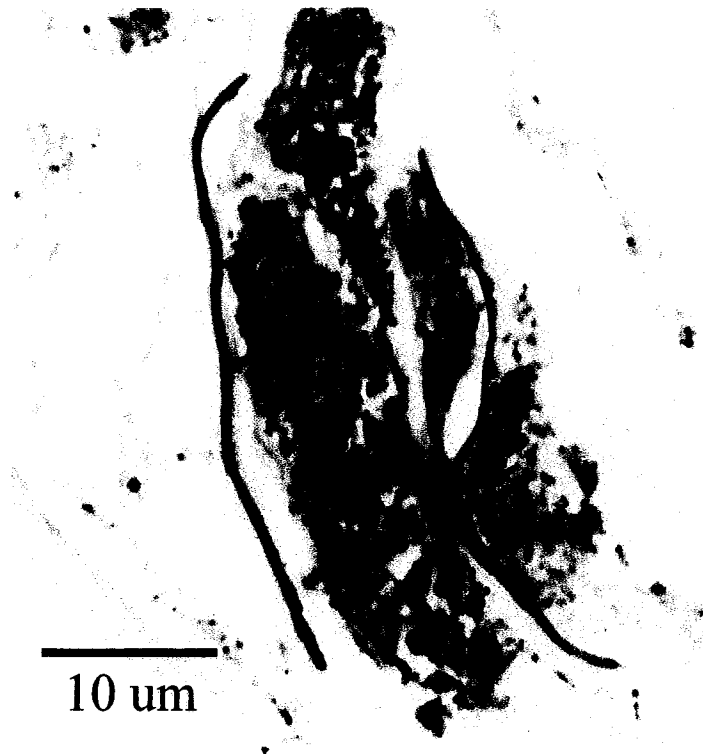
Figure 2.

10 um

Figure 3.

Timeline of Cholesteatoma Stages
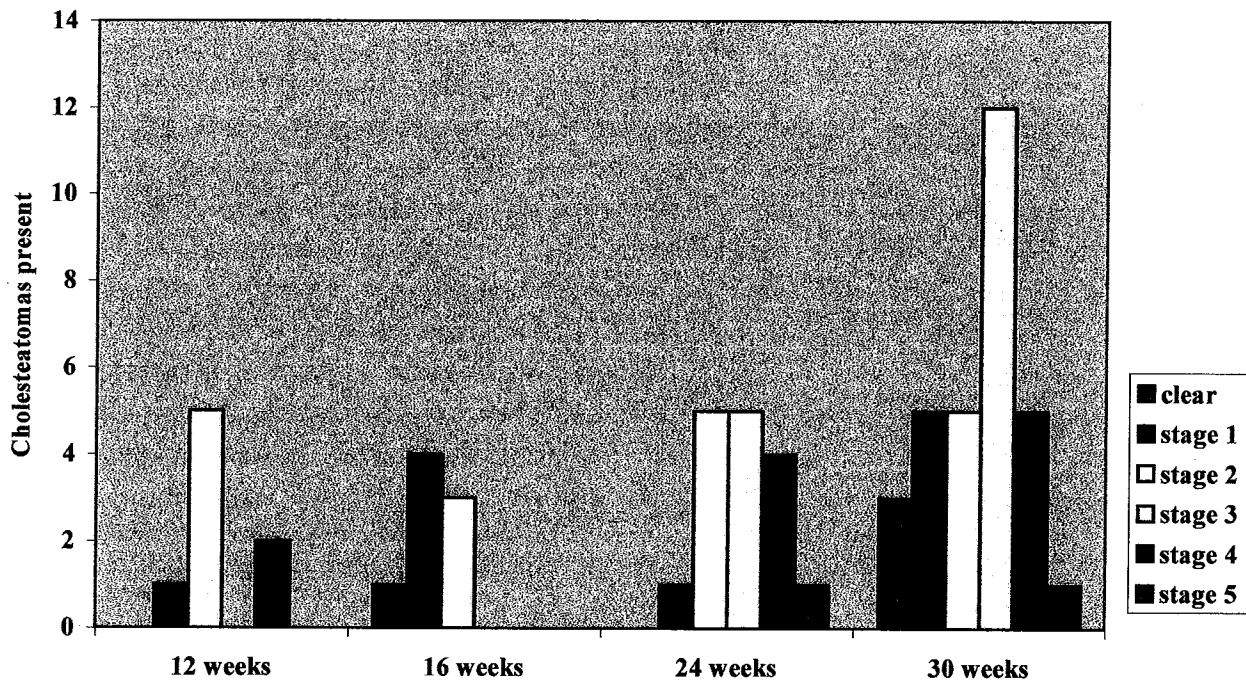
Figure 4.
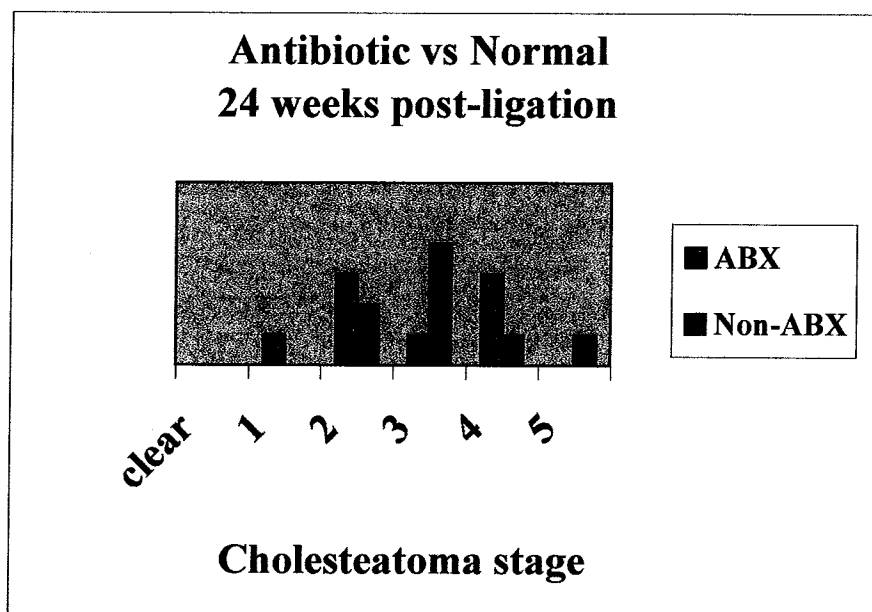
Figure 5.

Bacterial Isolate	% Occurrence	
	(Normal)	(ABX)
Proteus mirabilis +	65%	50%
Enterococcus faecalis- group D	75%	50%
Enterococcus casseliflavus	15%	0%
Enterococcus avium	5%	25%
Staphylococcus aureus *	60%	75%
Staphylococcus auricularis	55%	75%
Staphylococcus sciuri *	5%	0%
Staphylococcus saprophyticus *	5%	0%
Pseudomonas aeruginosa *	5%	25%
Enterobacter cloacae +	10%	0%
Escherichia coli *+	60%	75%

*found in Fulghum and Chole 1985
+found in Fortuny et al. 1993

Figure 6.

Bacterial Isolate	Weeks Post-Ligation																	
	12 wk				16 wk		24 wk											
							Antibiotic											
	1	2	3	4	6	8	9	10	11	12	13l	13r	14l	14r	15l	15r	16l	16r
Proteus mirabilis +	X	X	X	X	X	X		X	X					X	X	X	X	X
Enterococcus faecalis- group D	X	X	X		X	X		X	X		X	X	X	X	X	X	X	X
Enterococcus casseliflavus				X	X	X												
Enterococcus avium							X											
Staphylococcus aureus *	X	X	X	X			X	X		X	X		X	X		X	X	
Staphylococcus auricularis	X		X	X	X	X	X	X	X			X	X					X
Staphylococcus sciuri *		X																
Staphylococcus saprophyticus *				X														
Pseudomonas aeruginosa *										X								
Enterobacter cloacae +											X	X						
Escherichia coli * +		X			X			X	X	X		X	X	X	X	X	X	X

* Found previously by Fulghum and Chole 1985
+ Found previously by Fortuny et al. 1993

Figure 7.

Bacteria present at each cholesteatoma stage					
	stage 1	stage 2	stage 3	stage 4	stage 5
Proteus mirabilis	1	5	3 (1)	2	1
Enterococcus faecalis- group D	1	5	5 (1)	3 (2)	1
Enterococcus casseliflavus	1	1	0	1	0
Enterococcus avium	0	0	0	1	0
Staphylococcus aureus	0	3	5 (1)	4 (2)	0
Staphylococcus auricularis	1	3	2 (1)	5 (2)	0
Staphylococcus sciuri	0	1	0	0	0
Staphylococcus saprophyticus	0	0	0	1	0
Pseudomonas aeruginosa	0	0	0	0 (1)	0
Enterobacter cloacae	0	0	1	1	0
Escherichia coli	0	4	4 (1)	3 (2)	1
	1 ear	*5 ears*	*5 ears*	*6 ears*	*1 ear*

( )=cholesteatomas from antibiotic group

Figure 8.

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