SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of Pathomechanism and Therapeutic Possibilities of Inflammatory Ear Diseases

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Introduction

Inflammatory diseases of the ear are common pathological conditions both in Hungary and worldwide. General practitioners, pediatricians, emergency physicians, ENT specialists, neurologists and neurosurgeons may encounter inflammatory diseases of the ear, depending on the etiology, course and prognosis of the disease. Our research focuses on inflammatory diseases of the outer and middle ear. Several forms of inflammatory diseases of the outer and middle ear are known, out of which one form of infectious etiology was examined in relation to the middle ear and the outer ear respectively.

The development of microbiological diagnostic methods has broadened the microbiological profile appearing in ENT. The pathophysiology of diseases caused by already known pathogens was also revealed, and a better insight into the behavior of individual pathogens and the interaction of infectious agents was revealed. This opens new treatment options against various pathogens. We consider that it is important to know the exact pathomechanism of inflammatory diseases of the ear, since knowing this we are able to determine the most effective therapy.

Otitis externa affects 10% of people in their lifetime. Based on its course in time, it can manifest itself in acute or chronic form, and based on its localization and extent, it can manifest itself in diffuse or circumscribed form. In the background of the outer ear inflammation, typical conditions usually occur that erode the integrity of the skin of the external auditory canal. Regular swimming, humid environment, anatomically narrow auditory canal, eczema of the ear canal skin, hearing aid use and mechanical damage increase the risk of developing the disease. The particularly affected population is immunosuppressed or diabetic patients.

Candida *auris*, described as a new species, was isolated from the outer ear secretion of a 70-year-old Japan female patient in 2009. *C. auris* is an increasingly isolated multidrug-resistant yeast against which amphotericin B (AMB) is still the first choice of therapy in certain clinical situations such as meningitis, endophthalmitis and urinary tract infections. As an opportunistic pathogen, *C. auris* can be isolated worldwide, which can colonize skin and mucous membranes and cause life-threatening infections in critically ill patients. Using whole-genome sequencing, six phylogenetically distinct clades (South Asian, East Asian, South African, South American, Iranian and Singaporean) were identified, with significant genetic differences between the clades.

Previous studies in *Galleria mellonella* and neutropenic mouse models have shown that *C. auris* clades differ in virulence. *C. auris* is able to form large aggregates *in vitro* and *in vivo*, which phenomenon explains its excellent ability to survive under various environmental conditions, including antifungal treatment.

Otitis media is one of the most common ENT pathologies. Based on the duration of their existence and the course of their pathology, two large groups can be distinguished: acute and chronic forms. Acute otitis media can often become recurrent or progress to chronic inflammation. Inflammations take place through several stages that can be clearly distinguished based on the symptoms. The stages are called by their own names in literature. Resolutio can occur at any stage. It is influenced by etiology, the general and immunological condition of the patient, as well as the therapy used. In terms of prognosis, another possible direction of outcome is the transition to a chronic form. Chronic otitis media is divided into two main groups in literature. These inflammatory processes also develop through stages and can stop at each stage or reach the final stage, one of the chronic mesotympanal or chronic cholesteatomal otitis media, influenced by the factors mentioned in acute inflammation. If the acute or chronic inflammatory process goes beyond the confines of the middle ear due to lack of the body's defenses, anatomical or genetic predisposition, insufficient treatment or a combination of these, complicated otitis media may develop. Complications can remain outside the skull in which case we speak of an extracranial complication. Inflammations that spread intracranially are called intracranial complications. In our research, we examined patients in whom the process stops at the stage of chronic serous otitis media during the course of chronic inflammation. This condition is referred to in the literature as chronic serous otitis media (cSOM), but it can also be called glue ear or otitis media with effusion (OME).

Otitis media effusion (OME) is a condition with the presence of effusion in the middle ear (MEE) without the presence of acute signs of inflammation. This condition develops when the resolution of the acute process is incomplete, and the auditory tube cannot provide ventilation or continuous clearing (clearace) of the middle ear cavity.

The incidence of the disease reaches 90% in the first 6 years of life, peaking between six months and 4 years of age. The incidence of OME is higher in children with lower socioeconomic status. The disease is associated with hearing loss due to effusion in the middle ear. This symptom persists in 5-10% of patients and can lead to speech development disorders due to the resulting hearing loss. As the disease affects many children, it also places a significant economic burden on countries. In the United States, there are approximately 2.2

million hospital admissions per year due to OME, which has significant financial implications.

In its etiology, catarrhal diseases of the upper respiratory tract are important due to the blockage of the auditory tube, since in this way the ventilation of the middle ear cavity decreases. Subsequently, the activation of inflammatory cells provokes an increase in the permeability of the vessels in the middle ear mucosa. In childhood, the size of the components of the Waldeyer ring is proportionally larger relative to the size of the pharynx than in adults, so even a small enlargement of the adenoid tissue leads to blockage of the auditory tube orifice.

The diagnosis of the disease can be made by otoscopic examination and tympanometry. The otoscopic finding usually shows an eardrum where a straw-yellow effusion can be seen behind the eardrum with retained transparency, occasionally even air bubbles can be observed in it. With pneumatic otoscopy, a decrease in the mobility of the eardrum is also observed. Middle ear effusion is indicated by a type B "flat" curve in tympanometry.

During six months of observation, 50-60% of patients recover spontaneously. If spontaneous recovery does not occur, appropriate treatment should be considered: the patient's age, comorbidities, degree of hearing loss, duration of the condition and documentation of the sidelines. In Hungary, if there is no spontaneous recovery, adenotomy is performed, after which 6 weeks later we can assess the condition of the middle ear with a control hearing test. If the condition of the middle ear remains unsatisfactory, ventilation tube insertion is recommended. Adenotomy is necessary if effusion is caused by enlarged adenoids by blocking the opening of the auditory tube. The ventilation tube is necessary if ventilation of the middle ear is not solved by the removal of the adenoid. According to certain meta-analysis, adenotomy is more effective than ventilation tube implantation.

Objectives

Our research focused on two entities of inflammatory diseases of the ear: chronic serous otitis media (cSOM or OME: otitis media with effusion) and otitis externa induced by *Candia auris*.

In patients with chronic serous otitis media, the aim of the study was to demonstrate the concept that MALDI-TOF MS is a suitable and appropriate instrument to determine the

bacterial content of persistent middle ear effusion. Another goal was to use the new technology to gain insight into the bacterial spectrum of middle ear secretions in children with OME.

In the case of ear canal inflammation caused by *Candia auris*, the treatment of the inflammation caused by this pathogen is the most difficult problem to overcome. One reason for this is that data on *in vitro* killing activity against different *C. auris* clades were not yet available for the therapeutic products that could be used. Therefore, the aim of our work was to determine the killing activity of amphotericin B (AMB) against *C. auris* isolates belonging to the four most common clades.

Results of experiments with samples of middle ear effusion (MEE)

A total of 64 middle ear samples were analyzed from forty patients. Out of forty patients, 24 (60%) had bilateral OME. Fifty-nine samples (92%) were positive for at least one bacterium. A total of 39 bacterial species were identified and more bacteria were found in 29 samples (49%) of 23 patients (57%). Of these, only three – *Moraxella catarrhalis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* – were previously found to be pathogenic in OME. These three pathogens occurred in 12 samples from 11 patients and were often accompanied by other types of bacteria. No bacteria were found in two healthy middle ear samples from children who had undergone cochlear implantation.

OME has been extensively investigated for several decades. The principles of etiology were established in the 1960s. It was considered that auditory tube dysfunction should play a fundamental trigger role in the development of the disease. Later, the etiology of OME was gradually supplemented by new theories. Bacterial and viral inflammatory effects, impaired mucociliary function, IgE-mediated allergic reaction and atopy, laryngopharyngeal reflux, inadequate host response due to inflammatory mediators and receptor polymorphism and recently the presence of bacterial biofilm have appeared as etiological factors, although some of these are still disputed. Currently, OME is considered a multifactorial immunological disease. As our study highlights the importance of the presence of multiple bacteria, it will be interesting to explore how this presence is causally related to various deficient immune functions and other anatomical and physiological parameters of the middle ear, such as insufficient ventilation due to the impaired auditory tube function or 3D morphoanatomy.

Anamnestic data were the most important for patient selection during admission, as the disease is not expected to be accompanied by acute signs and symptoms. The simplest and cheapest diagnostic tool available for checking fluid in the middle ear is pneumatic otoscopy. It has the best balance between sensitivity and specificity. Despite its higher financial and administrative burden, tympanometry is a reliable diagnostic tool in routine clinical practice. Since objective documentation was essential for the examination, the latter diagnostic method was used.

According to recent guidelines, OME should be treated only if symptoms persist for more than 6 months and do not go away spontaneously. Significant hearing loss or suspected structural abnormalities of the eardrum or middle ear are also conditions that require treatment for OME. In recent decades, many therapeutic strategies have been tried, but most of them have proved ineffective. The abundant bacterial flora of MEE suggests that bacterial interference may play a role in the long-term persistence of the disease.

Middle ear discharge can be accurately examined using MALDI-TOF MS. This method allows the simultaneous study of more than two thousand species of bacteria, making it easy to identify a huge, diverse and abundant bacterial flora. The bacterial pattern of middle ear secretions resembles the flora of acute otitis media (AOM), although more types of pathogens and other bacterial species were identified in the current study than in previous studies. In addition, only a subset of the samples tested positive for *M. catarrhalis*, *H. influenzae* and *S. pneumoniae*, pathogens previously thought to contribute to OME. Most of the samples did not contain these bacteria, but other bacteria were identified in them. Some of them play an obvious pathogenic role in other types of infections, others are non-pathogenic or facultative pathogens.

Results of experiments with *C. auris*

Regardless of clades and isolates, MIC values obtained by standard Broth microdilution (BMD) and E-test methods (after 24 and 48 hours, respectively) were not higher than sensitivity limit (1 mg/L) recommended by the CDC. In MFC studies determining the minimal fungicid concentration AMB was shown to be fungicidal against the 4 most prevalent clade of C. *auris* after 24 hours in the range of 1-8 mg/L; MFC values were 2-32 times higher than MIC values obtained by BMD. Importantly, apart from isolate 20 (South Asian clade), these MFC values were higher than clinically available serum AMB concentrations (1 mg/L). In our time-kill experiments, AMB showed concentration-, clade- and isolate-dependent killing activity in *C. auris* against isolates. MFC results showed good correlation with time-killing results, except for isolates 12372 (East Asian clade), 228 (South African clade), and I-172 (South American clade).

AMB was found to be fungicidal at clinically available 1 mg/L for two out of six isolates from the South Asian clade, two out of four for the East Asian clade, three out of six for the South African clade, and one out of six for the South American clade. In addition, of the 6-6 isolates from the South Asian and South American clades, two and three isolates showed regrowth at 1 mg/L, respectively. With wide-angle fluorescence microscopy, a decrease in the number of cells in isolates 196, 15 and 228 was detected, while large aggregates were found at 1 mg/L in isolate I-156 (Figure 6), but dead cells were never detected. Based on our results, at clinically achievable concentrations in serum (≤1 mg/L), AMB may not be fungicidal against any of the investigated *C. auris* clades.

Limited data are available on *in vitro* killing activity of AMB against *C. auris*; Nine Colombian bloodstream isolates were previously studied. In this study, AMB was fungicidal in MFC tests at 2-4 mg/L (1-4xMIC) and showed concentration-dependent but isolate-independent killing activity at >2 mg/L in time-kill assays. T99.9 time-ranges (time required for reducing the number of fungi by 99,9%) were significantly longer (between 3.3 and 11.7 hours, the right k values ranged from 0.256 to 0.913 1/h) than T99.9 values (at 2 mg/L) of our 4 isolates in our own studies. However, in this previous study T99,9 values were based on an averaged k value, i.e. single k represented the killing kinetics in the range of 0.12-8 mg/L for each strain. It is noteworthy that for our remaining two isolates (I-24 and I-156) no fungicidal effect was observed at 2 mg/L.

Due to the lack of preliminary studies on AMB killing activity against isolates from clades in South Asia, East Asia and South Africa, it was not possible to compare with independent results.

Unfortunately, only two isolates (from the East Asian clade) came from patients with otitis externa. However, in our studies, we defined the AMB *in vitro* killing activity against a large number (22 isolates) of C. *auris* strain belonging to the four main clades, which is considered the main strength of our work. These results were confirmed *in* v*ivo* in neutropenic mouse model. Other studies showed that 5 mg/kg AMB treatment resulted mainly fungistatic (8 out of 9 isolates) effect against *C. auris* isolates in nine of the four clades, and in one case the number of fungal cells cultured from the kidneys increased despite AMB treatment. Interestingly, only 3 cases of the 9 examined isolates achieved a 1-log reduction. The number of fungal cells cultured from the kidneys of mice infected with isolates with high AMB MIC values (2-4 mg/L) increased despite AMB treatment.

Based on our results, at clinically achievable concentrations (≤1 mg/L), AMB was fungicidal against 16.7-50% of isolates from the four geographical clades. The background of AMB's poor killing activity against our non-AMB resistant isolates is unknown. Mutations in ergosterol biosynthesis genes have been previously reported in such isolates, but not all AMB resistant isolates showed such mutations. In another study, no mutations were found in Colombian AMB resistant *C. auris* isolates in the genes of ergosterol biosynthesis. Four alternative AMB resistance mechanisms have been described, including mutations in genes coding for transcription factors like FLU8 in yeasts and a hypothetical membrane transporter. Another possible mechanism is that changes in cell wall components, specifically β-1,3-glucan or chitin, can physically reduce AMB entry into cells. The lowered penetration of AMB into *C. auris* cell was supported by our I-156 isolate, which formed large aggregates in the presence of 1 mg/L AMB. Although clinical relevance remains unknown, it is noteworthy that the two more AMB resistant clades (South Asian and South American) were the most virulent in neutropenic mouse models in our previous studies.

In summary, the MFC and our time-killing results highlight the poor killing activity of AMB against the *C. auris* isolates, regardless of clade, even if the MIC value is low (≤1 mg/L). These data suggest that the efficacy of AMB in invasive *C. auris* infections (meningitis, endophthalmitis and urinary tract infections), cannot be reliably predicted based on MIC values, which may explain the high mortality observed during AMB treatments. However, the combination of echinocandins and AMB may improve survival.

Summary

Our study shows that MALDI-TOF MS is suitable for evaluating the bacterial composition of middle ear secretions in children with chronic serous otitis media. Although in the current approach this technique only detects bacteria that can be cultured, it improves our ability to detect a wider spectrum of bacterial species compared to traditional bacterial culture approaches and is able to identify species. The presence of bacteria in OME is obvious. Our study suggests that for OME, multispecies bacterial flora may play a central role in the persistent presence of fluid in the middle ear, or it may be a natural and obligatory accompanying phenomenon of OME. This previously undescribed bacterial invasion can contribute to proper understanding and treatment of OME.

Summarizing the experiments with *C. auris*, our MFC and time-kill results draw attention to the weak killing activity of AMB against *C. auris* isolates regardless of clade, even if the MIC value is low (≤1 mg/L). These data suggest that the efficacy of AMB in treating invasive *C. auris* infections, including meningitis, endophthalmitis, and urinary tract infections, cannot be predicted based on MIC results, which may explain the high mortality during AMB treatment. Although the combination of echinocandins and AMB may improve survival, the discovery of new antifungal agents with conventional or new targets is essential to improve survival rates for multidrug-resistant fungi, including *C. auris* infections.

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A PhD értekezés alapjául szolgáló közlemények

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