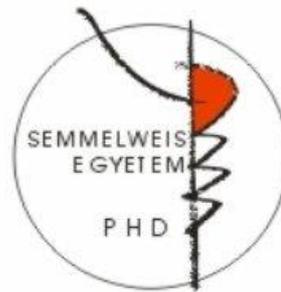


In vitro microbiological testing of experimental Drug Delivery System

PhD thesis

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Introduction

Foreign bodies in human body are known as a special problems. Prosthetic joint, prosthetic valve, central venous catheter, peripheral venous canule, nephrostoma or urinary catheter have the ideal surface for the colonisation of microbes. Central venous catheters is a good possibility for the microbes to penetrate continuously into the body. Infection of prosthetic joints can cause abscesses at the local tissues. Following the local infection, microbes can be transported to the different organs by the circulation forming septic metastases and cause bloodstream infection (BSI).

Latent infections in the human body can cause prosthetic joint infections (PJI) quite frequently. From these sources of infections, microbes can be also transported by the bloodstream and colonise the surface of the prosthetic. Usually these microbes come from the nosocomial flora as well. Poly-methyl-metacrylate (PMMA) bone cement widely used for the fixation of prosthetic can cause necrosis in the bone tissue by elevating the temperature and damaging the capillaries. In the damaged tissues, microbes can cause pyogenic discharge and consequently osteomyelitis can be formed in local bone tissues. These serious event create the ability of recurrent sepsis or prosthetic joint can lose of function in the following years.

Strains causing PJIs can form adhesive factors which can help the bacteria attaching on the foreign surfaces and forming biofilm. PMMA can behave as a receptor of glycocalyx produced by the bacteria. Among Gram-positive bacteria, *Staphylococcus epidermidis* can form biofilm most frequently on the prosthetic surface. Among Gram-negative ones, *Pseudomonas aeruginosa* is the most important biofilm forming bacteria.

From the 1950s, coagulase-negative Staphylococci (CNS) strains have been taken an important role in foreign body associated infections. The number of cases caused by CNS strains started to increase, and after 2000, most of the nosocomial infections caused by these bacteria in certain European and American hospitals. In present days, *S. epidermidis* is one of the most dominant pathogen in nosocomial infections, but the importance of Gram-negative strains is also elevated.

The widely used beta-lactam antibiotics contributed to the spread of methicillin resistant *Staphylococcus epidermidis* (MRSE) strains. These strains are more invasive than the methicillin susceptible *Staphylococcus epidermidis* (MSE) strains, and these bacteria can be responsible for the deep-prosthetic joint infections (DPJI). MRSE strains usually show reduced

susceptible to glycopeptides and numerous studies have been highlighted on the elevated resistance to aminoglycosides in West Europe (more than 60%).

Nowdays, systemic antibiotic therapy can poorly cure the prosthetic joint infection, because the proliferation of microbes under the biofilm can not be influenced in that way. Therefore, a local therapeutic method is necessary to use to prevent and cure septic discharges of prosthetic joint. The local antibiotic releasing systems called as Drug Delivery Systems (DDSs) are devided in two groups: none-biodegradable and biodegradable systems. From the 1980s, in orthopedic surgery, none-biodegradable systems, as gentamicin containing Septopal-chains and gentamicin-tobramycin impregnated bone cement have been widely used. Nowdays, the elevating resistance to aminoglycosides limitates the usage of these systems and makes us to develop new, biodegradable systems. The advantage of biodegradable system is, that after releasing the antibiotic, that one can be desintegradet by human body and it is not necessary to remove that one by reoperation. Newly developed systems contains vancomycin, teicoplanin or amikacin, because these agents can eradicate beta-lactame resistant *S. epidermidis* strains.

Research objectives

My objectives were:

- the retrospective epidemiological investigation of pathogens caused prosthetic joint infections between 2001 and 2011 at Orthopedic Clinic of Semmelweis University
- to investigate the growing kinetic of *Staphylococcus epidermidis* in different liquid culture media
- to demonstrate the antibacterial effectivity against *Staphylococcus epidermidis* strain of two different, wax-based drug delivery systems containing different concentrations of vancomycin.
- to investigate the drug release profile of two different, wax-based drug delivery systems containing different concentrations of vancomycin.
- to investigate the biofilm formation of *Pseudomonas aeruginosa* strains.
- to find the certain synergistic antibiotic combinations, which are effective against *Pseudomonas aeruginosa* strains and inhibit the biofilm formation.

Material and methods

Data collection

We collected the clinical data of prosthetic joint infections from Orthopedic Clinic of Semmelweis University between 2001 and 2011. The culture results of clinical specimens were registered at Clinical Microbiology Diagnostic Laboratory of Semmelweis University.

Processing of microbiological specimens

The clinical specimens were transported by transport media (Amies Agar Gel, 108C, Copan Diagnostics Inc, USA) to the laboratory. The storage of the strains were performed in tryptic soy broth culture media (TSB) on -80 °C. Before the experiments, bacteria were recultivated.

Bacterial strains

For the investigation of drug release and growing kinetic, we used *S. epidermidis* ATCC 35984 laboratory strain. As control strain, *Bacillus subtilis* ATCC 6633 (Becton-Dickinson, USA) were used. For the biofilm formation tests, 60 non-related *P. aeruginosa* strains were collected from the Microbiology Laboratory of Medical Centre, Hungarian Defence Forces. Furthermore, as positive control, *Staphylococcus aureus* ATCC 12600 and as negative control, *S. epidermidis* ATCC 12228 strain was used (LGC Standards GmbH, Germany).

Determination of minimal inhibitory concentration

According to the recommendation of Clinical and Laboratory Standards Institute (CLSI) and National Committee Of Clinical Laboratory Standard (NCCLS), determination of minimal inhibitory concentration (MIC) of different antibiotics were presented by microdilution method. These antibiotics were piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, gentamicin, amikacin, netilmicin, tobramycin, ciprofloxacin, levofloxacin, chloramphenicol.

Determination of the number of bacteria

We determined the number of bacteria by dilution method in different broth media. The initial number of colony forming units (CFU) were determined by McFarland measurement.

Determination of vancomycin concentration in different liquid culture media

For determination vancomycin concentration in different broth media, we used biological, filter paper method. We analysed the correlation between the diameter of the of inhibition zone

around the filter paper disc and dilution row of vancomycin concentration ($\mu\text{g/ml}$). The different liquid culture media were the following ones: brain-heart infusion (BHI), thyoglycolate (TG), Müller-Hinton broth II (MH broth II) and saline solution.

Investigation of the growing kinetic of *Staphylococcus epidermidis*

The growing kinetic were analysed by dilution method. The maximal dilution was 10^5 CFU/ml. At final dilution, we inoculated $10\mu\text{l}$ suspension on MH culture media, and after incubation period, we counted the colonies.

Investigation of antibacterial effect of wax discs.

We used Wax1 (Cera alba : Precirol® : Vaselinum album 45 : 45 : 10 composition) and Wax2 (Cera alba : Precirol® 50 : 50 composition) discs with different vancomycin content (0.5, 1, 2 and 4 mg). The antibacterial effect of wax discs were analysed by dilution method BHI media and presented by time-kill curves.

Investigation of the drug release of wax discs on solid culture media

The drug releasing kinetics from DDS were demonstrated by Bauer-Kirby method on solid MH culture media.

Investigation of the drug release of wax discs in liquid culture media

The drug releasing kinetics from DDS were demonstrated in BHI culture media by biological method with filter paper discs. We tested these filterpaper disc on the culture of *B. subtilis* ATCC 6633 strain.

Investigation of biofilm formation

We used a qualitative method for biofilm screening. The bacteria were cultivated in TSB overnight at $37\text{ }^\circ\text{C}$ in U-wells of polystyrene microtiter plates. The wells were washed three times with phosphate-buffered saline and dried in the inverted position, than we died the plates with safranin. The well-visible film lining of the well walls was considered as positive for biofilm production.

Biofilm susceptibility testing

Bacteria were cultivated in U-wells of polystyrene microtiter plates with the appropriate dilutions of the respective antibiotics in MH broth II and the plates were incubated for 18–20 h

at 37 °C. The wells were washed three times with phosphate-buffered saline and dried in the inverted position. MBICs (the lowest concentration of antibiotic which inhibits biofilm formation; µg/l) were then determined. The strains were tested for susceptibility to certain antibiotics mentioned at MIC determination method.

Biofilm synergy testing

Serial twofold dilutions of each drug to at least double the MIC were prepared. MH broth II was distributed into each well of the microdilution plates. The first antibiotic of the combination was serially diluted along the ordinate, while the second drug was diluted along the abscissa. An inoculum equal to a 0.5 McFarland turbidity standard was prepared from each *P. aeruginosa* isolate in MH broth. After the inoculation of bacteria, incubation period followed. After washing and dyeing, well-visible film lining of the well walls was considered as positive for biofilm production. The resulting checkerboard contains each combination of two antibiotics, with tubes that contain the highest concentration as control. FBIC of each agent was calculated as follows:

$$FBICA = MBICA(c) / MBICA(a) \quad FBICB = MBICB(c) / MBICB(a)$$

$$\Sigma FBIC = FBICA + FBICB$$

where subscripts A and B denote antibiotics A and B, subscripts in parentheses c and a denote the activity measurements in combination and alone, respectively.

The summation of both FBICs was used to classify the combination of antimicrobial agents as synergistic ($\Sigma FBIC \leq 0.5$), partially synergistic ($0.5 < \Sigma FBIC \leq 1$), indifferent ($1 < \Sigma FBIC \leq 4$), or antagonistic ($\Sigma FBIC > 4$).

Statistical analysis

In different liquid media, the growing kinetics were compared with correlation coefficient. For killing curve study the General Linear Model (GLM) was used and to compare each group Fisher LSD test was used, taking in both cases a *p* value of 0.05 as significant. To compare the initial/early stage of the vancomycin release also the GLM and to compare the CFUs decreasing in each liquid media two tailed t test was used for statistical analysis taking a *p* value of 0.05 as significant and to compare the peak vancomycin concentrations the two-way ANOVA test was used, taking a *p* value of 0.05 as significant.

Results

Retrospective epidemiological investigation of prosthetic joint infections

We investigated 221 culture results between 2001 and 2011. Gram-positive bacteria caused 64 % of the PJIs. 53 % of the PJIs were caused by Staphylococci. *S. epidermidis* caused most of these cases (28%) and *S. aureus* caused 25 % of these infections. *P. aeruginosa* stains were responsible for 8 % of the PJIs.

Dermination of minimal inhibitory concentration of different antibiotics

The average of MIC values of 12 antibiotics tested to 14 *P. aeruginos* strains are summarised in Table 1.

Table 1: Average of the MIC values (aMIC) of 12 antibiotics.

Antibiotics	aMIC values ($\mu\text{g/ml}$)		
	MIC ₅₀	MIC ₉₀	Range
Piperacillin/tazobactam	1024	1024	128–1024
Ceftazidime	4	32	2–1024
Cefepime	8	32	2–64
Imipenem	4	16	0.5–128
Meropenem	16	32	2–64
Ciprofloxacin	256	512	8–1024
Levofloxacin	32	62	2–256
Amikacin	4	32	2–1024
Gentamicin	128	256	1–256
Tobramycin	128	512	2–>1024
Netilmicin	4	>256	1–>256
Clarithromycin	256	>1024	32–>1024

Determination of vancomycin concentration in different liquid culture media

In BHI and MH broth II media, 2 $\mu\text{g/ml}$ vancomycin concentration caused 18 mm diameter of the inhibition zone. In saline solution this concentration formed 19 mm diameter of the inhibition zone. There was no inhibition zone at 0.125 $\mu\text{g/ml}$ vancomycin concentration in TG culture media. In BHI és MH broth II media, more than 10 mm diameter of the inhibition zone was formed. At 0.25, 0.5 és 1 $\mu\text{g/ml}$ vancomycin concentration, the diameter of the inhibition zone was between 10 and 18 mm in all culture media.

Investigation of the growing kinetic of *Staphylococcus epidermidis*

There was a strong, tight, positive correlation between CFU reducing kinetics in MH broth II and TG liquid culture media compared with saline solution. ($r_{\text{saline, MH}} = 0,96$, $r_{\text{saline, TG}} = 0,93$). Strong, positive correlation was observed between BHI media and saline solution ($r_{\text{saline, BHI}} = 0,89$). There was no initial growing phases of *S. epidermidis* at 2, 4 és 8 $\mu\text{g/ml}$ vancomycin concentration and after four hours long constant period, there was strong decreasing in MH broth II. In saline solution, the declination phase was close to linear and started earlier. In control BHI containing no vancomycin, we detected the most intensive multiplication. Without vancomycin the count of bacteria reduced below the initial count of CFU after 24 hours in saline solution.

Investigation of antibacterial effect of wax discs.

There was a permanent increase of the bacterial count in the group containing no Wax. There was a permanent decrease of the bacterial count in the other groups. Wax2 with 4 mg vancomycin presented the strongest antibacterial effect. After two hours, the initial 9.9 log₁₀ CFU/ml was reduced below 7 log₁₀ CFU/ml, and there was no effect like this at other groups. None of the groups presented initial growing phase. Wax2 with 4 mg vancomycin showed the strongest decreasing of the initial bacterial count after four hours in BHI comparing to the other liquid media containing no Wax. Wax1 and Wax2 containing 1 and 2 mg vancomycin reduced significant the initial bacterial count only after six hours. The same decreasing happened at Wax1 and Wax2 containing 0.5 mg vancomycin after 24 hours (Figure 1).

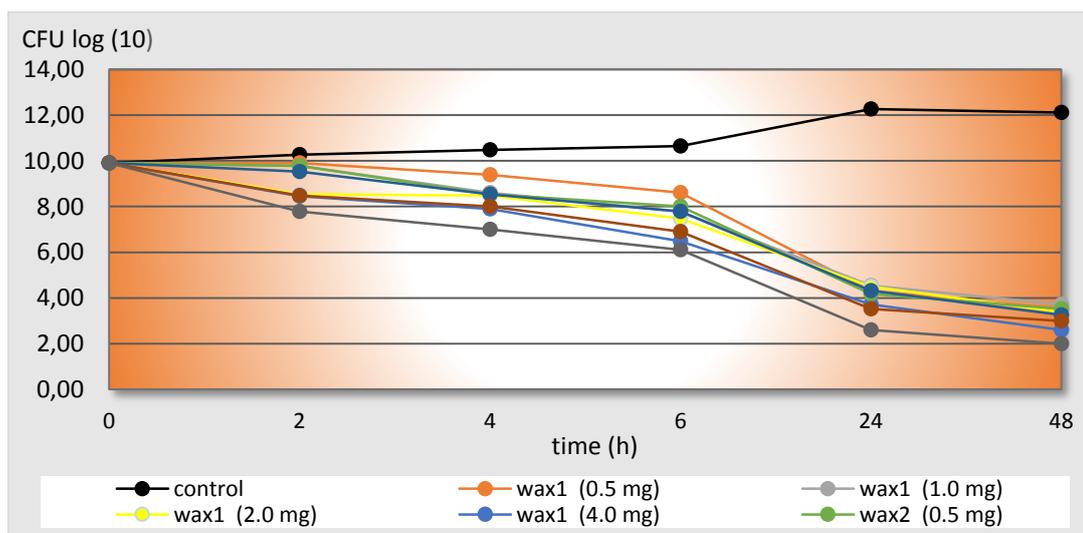


Figure 1: Decreasing of initial bacterial count at different Wax-based systems (Time-Kill Curves).

Investigation of the drug release of wax discs on solid culture media

There was no inhibition zone on the culture of *S. epidermidis* at Wax1 and Wax2 containing 0.5 mg vancomycin. Wax-based system containing 1 mg or more amount of vancomycin formed more than 18 mm diameter of the inhibition zone after incubation period (Figure 2 and 3).

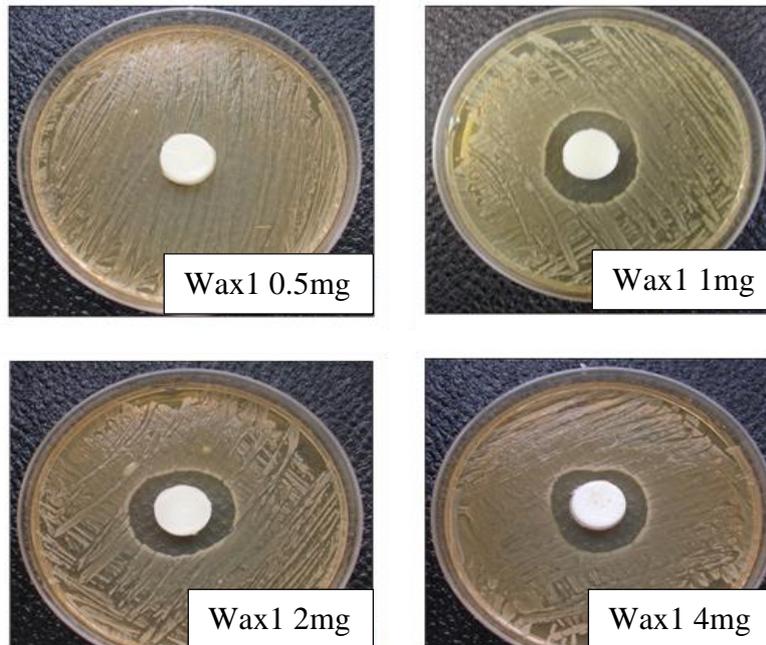


Figure 2: Inhibition zones of Wax1 containing 0.5, 1, 2 és 4mg vancomycin after overnight incubation.

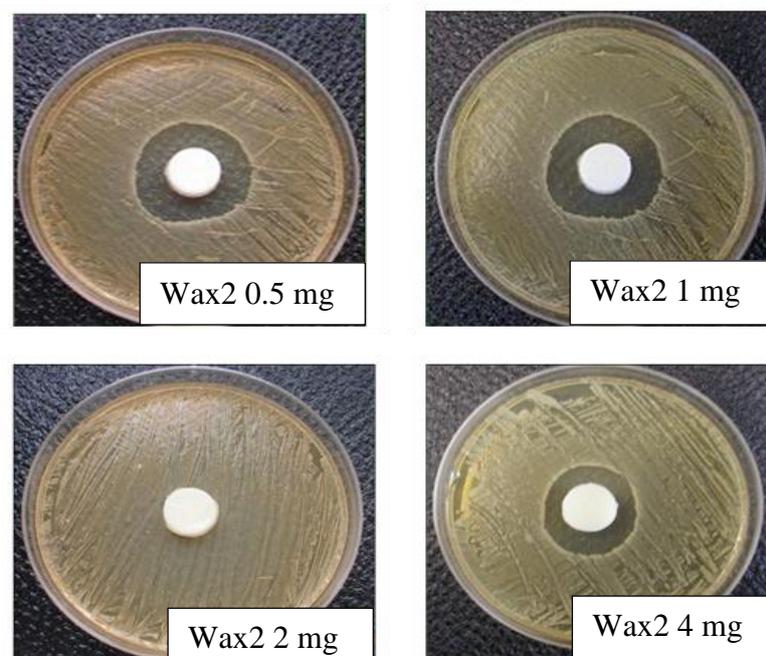


Figure 3: Inhibition zones of Wax2 containing 0.5, 1, 2 és 4mg vancomycin after overnight incubation.

Investigation of the drug release of wax discs in liquid culture media

In general the vancomycin started to dissolve significantly ($p = 0.0134$) earlier in the case of the Wax2 than Wax1. Furthermore, the start of vancomycin dissolve depended also on the vancomycin content of the waxes, thus the dissolve started earlier in the presence of the increased vancomycin content. There was a significant correlation ($p = 0.000001$) between the incubation time and vancomycin concentration. The observed peak vancomycin concentration increased by the used vancomycin content, it is the lowest ($0.25 \mu\text{g/ml}$) in the case of Wax1 with 0.5 mg vancomycin content and the highest ($1 \mu\text{g/ml}$) in Wax2 with 4 mg vancomycin. Differences were observed in the case of vancomycin content comparing the 72 hours peak concentrations but, there was no significant differences ($p = 0.0914$) between the Wax1 and Wax2 groups. However, there was significant difference ($p = 0.0184$) in the peak concentrations between the 4 mg vancomycin containing group and all the other groups. Comparing the groups with $0.5, 1$ and 2 mg vancomycin content, no significant differences was observed between the other groups ($p = 0.1675$). According to the decreasing of initial bacterial count, Wax2 with 4mg vancomycin showed a strong, positive correlation to different liquid culture media containing free vancomycin, and this correlation was very strong to saline solution (Figure 4).

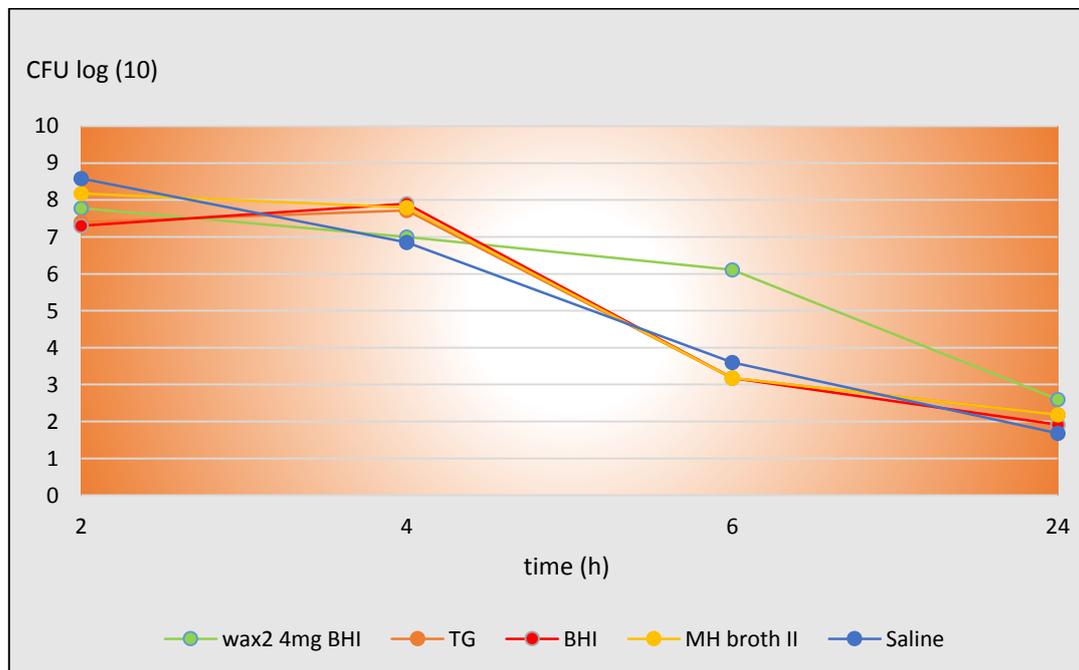


Figure 4: Comparison of Wax2 with 4 mg vancomycin and $1 \mu\text{g/ml}$ free vancomycin concentration in liquid culture media according to the decreasing of initial bacterial count.

Investigation of biofilm formation

A total of 14 (23.3%) isolates out of 60 isolates of *P. aeruginosa* were positive for biofilm production. The qualitative method for biofilm screening is presented on Figure 5.



Figure 5: Biofilm screening method. The purple colorisation on the bottom of U-wells shows the biofilm formation.

Biofilm susceptibility testing

Three antibiotics themselves were proved to be efficient biofilm production inhibitors: meropenem, piperacillin/tazobactam and clarithromycin, they had the lowest MBIC₉₀ values. For biofilm-forming *P. aeruginosa* strains 2-fold to 128-fold higher MBIC values than MIC values were obtained for ceftazidime, cefepime, imipenem, amikacin and netilmicin. The MBIC was 2-fold to 512-fold lower than the MIC values in the case of piperacillin/tazobactam, ciprofloxacin, levofloxacin and clarithromycin. Comparison of MIC and MBIC values are presented on Figure 6.

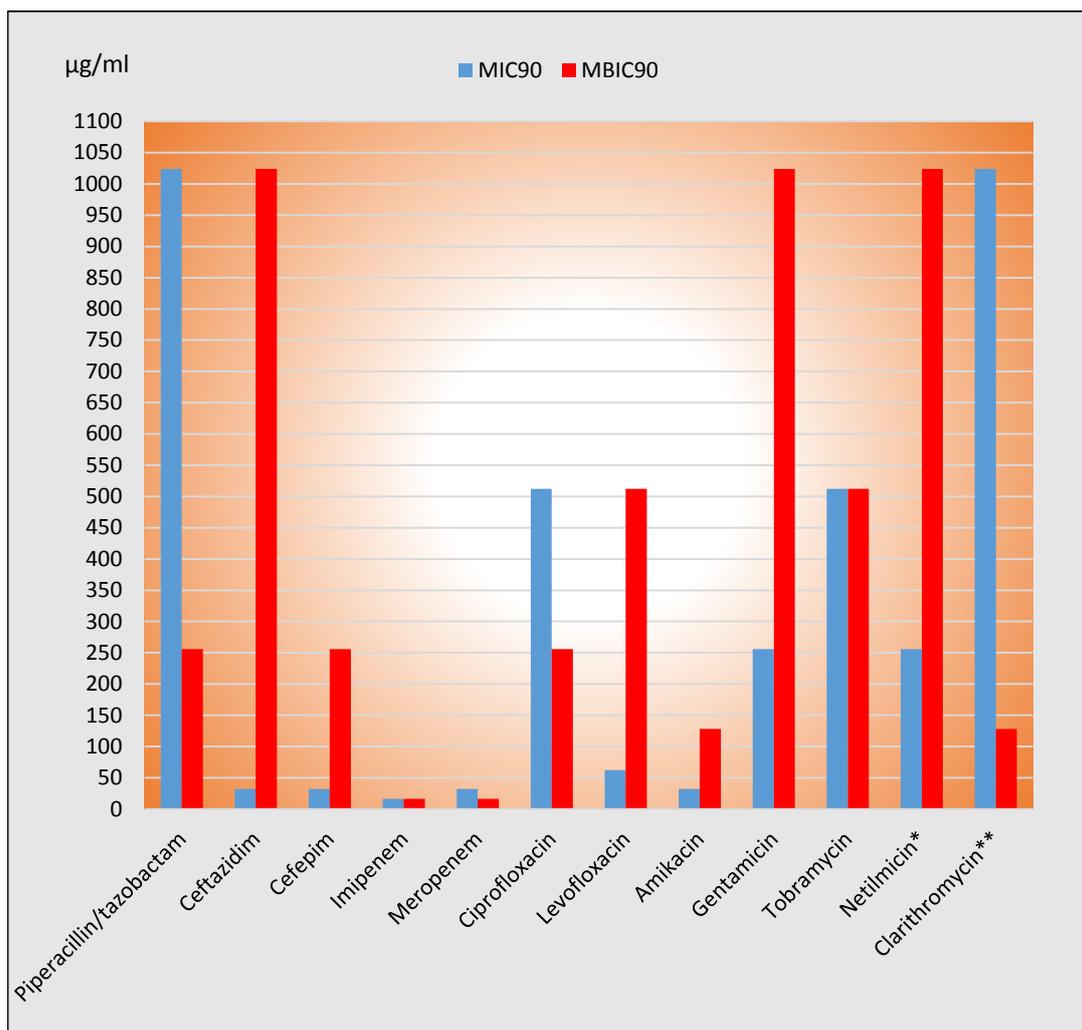


Figure 6: Comparison of the MIC és MBIC values of tested antibiotics to biofilm producing *P. aeruginosa* strains.

Biofilm synergy testing

On eight *P. aeruginosa* isolates were performed the synergy test (13,3%). Apart from some cases the fluoroquinolones had low FIC-indexes in combination with clarithromycin. Clarithromycin-levofloxacin combination was proved to be synergistic at 5 of the 8 strains (62.5%). Ciprofloxacin was synergistic with the macrolide at half of the strains, and so the ceftazidime. The aminoglycosides were mainly synergistic with the clarithromycin, and if so, they had very low FIC-index values. The synergy was generally demonstrated for clarithromycin and aminoglycosides, fluoroquinolones and ceftazidime. However, antagonism was detected for clarithromycin and meropenem and cefepime in 62.5% and 75% of the investigated biofilm-forming *P. aeruginosa* strains. The ceftazidime, the fluoroquinolones and

aminoglycosides were highly effective against most of the strains in combination with clarithromycin. The carbapenems were mostly ineffective in combination with clarithromycin.

Discussion

Certain limitations of the epidemiology investigation are revealed during our study. In European countries, only a few surveillance investigations were presented in the past years, therefore it is very difficult to compare our results to result of European medical centers. Furthermore, in Hungary, many hospitals use not to take part in annual surveillance programe, so our results can not be compared with them. Anyway, results of our epidemiological investigation shows correlation with European data in general. Our rate of the PJIs caused by *S. epidermidis* is nearly the same as in foreign medical centers. *S. aureus* was isolated less than in orher hospitals, but Gram-negative bacteria caused more sepsis in connection with prosthetic joint.

For the investigation of DDS, it was necessary to determinate the suitable liquid media, which can imitate the physiological environment of human tissue. We tested three enriched liquid culture media (BHI, TG, MH broth II) and saline solution as control. Theese liquid media did not influenced the bactericidal effect of vancomycin. In literature, BHI is the widely used culture media because this one is very rich in nutrients. Saline sollution can keep the starting number of *S. epidermidis*, but bacteria can not multiply because of the lack of nutrients.

In orthopedic surgery, different kind of antibiotic carrier systems were developed in the past years. These carriers are needed to remove after depletion of antibiotic and cannot be used as vitalisable tissue material. Removing of these carriers menas the possibility of infection. Avoiding the reoperation, our team were be able to develop an antibiotic carrier system for preventing the prosthetic joint infections. Our DDS made by wax can be degradable by macrophages after releasing the antibiotic. Cadaver bone tissue, which is widely used in ortopedic surgery can be impregnated with wax, and in that way, theese system can be used as replacement of damaged, infected bone tissue paralelly releasing vancomycin. Vancomycin has a multiple effect againts *S. epidermidis*, such as the inhibition of cell wall synthesis, which could happen only during the multiplying period.

According to the former studies, 2 µg /ml coccentration of vancomycin presents the MIC concentration to widely used ATCC 35984 *S. epidermidis* control strain. In our study, this concentration was caused a rapide bacteria killing effect. Lower concentration of vancomycin was also enough to reduce the number of CFUs for six days. At 1 µg /ml vancomycin concentration, the initial count of bacteria was reduced continously in all kind of liquid media.

Wax1 and Wax2 containing 1 mg or more vancomycin could maintain the 18 mm diameter of inhibition zone for six days. Based on our result the Wax 2 with 4 mg vancomycin content had the highest peak concentration in liquid media equal to the minimal inhibitory concentration of *S. epidermidis* ATCC 35984 and the Wax 2 with 4 mg vancomycin content had the best antibacterial effect, so it could be a potential agent for clinical use in the future. This type of wax disc can maintain 1 µg /ml vancomycin concentration in physiological environment. According to certain studies, only rifampicin and amikacin has the same strong bactericidal effect as the vancomycin.

Qualitative biofilm screening test is an easy, cheap test to verify biofilm formation avoiding the performance of molecular biological methods. Macrolides may also be beneficial in *Pseudomonas* infection, even if they have limited direct antibacterial activity against *P. aeruginosa*. Macrolides have been shown to reduce the biofilm production *in vitro* because of inhibiting the quorum sensing mechanism. Various reports on the impact of clarithromycin on biofilm destruction are available, but it was also reported that clarithromycin has no bactericidal action on biofilm synthesis or on pre-formed biofilms. However, some bactericidal effects could be demonstrated in this study. Ceftazidime combined with fluoroquinolones has a strong synergistic effect to inhibit biofilm. Clarithromycin combined with fluoroquinolones and aminoglycosides show also a strong synergistic effect against biofilm formation. Aminoglycoside combined with normally anti-*Pseudomonas* agents, such as cefepime, antagonistic effect was performed.

Conclusions

1. In our investigation, the rate of PJIs caused by *S. epidermidis* is nearly the same as in European medical centers. Nowadays, the number of Gram-negative bacteria caused sepsis in connection with prosthetic joint is elevated.
2. BHI, TG and MH broth II can be used for in vitro testing, because they do not influence the effect of vancomycin. BHI is the widely used culture media because this media is very rich in nutrients. Saline solution can keep the starting number of *S. epidermidis* but bacteria can not multiply in that media, so it is not suitable for in vitro tests.
3. Wax 2 with 4 mg vancomycin content has the best antibacterial effect, so it could be a potential agent for clinical use in the future. This type of wax disc can maintain 1 µg /ml vancomycin concentration in physiological environment, which is also enough to reduce bacterial count.
4. Wax 1 and Wax 2 containing 1 mg or more vancomycin can maintain the 18 mm diameter of the inhibition zone for six days. Controlled releasing of antibiotic from Wax 2 with 4 mg vancomycin can maintain 1 µg /ml vancomycin concentration in physiological conditions for 1 week. With this continuously presented concentration of vancomycin, septic discharge of prosthetic joint can be prevented and osteomyelitis also can be treated parallel with parenteral administration of antibiotics.
5. Qualitative biofilm screening test is an easy, cheap test to verify biofilm formation and it is not necessary to do molecular biological test if we are not interested in the genetic background of biofilm formation.
6. Ceftazidime combined with fluoroquinolone antibiotics can highly inhibit the bacterial biofilm produced by *P. aeruginosa*. Clarithromycin can be well combined with different antibiotics such as fluoroquinolones and aminoglycosides to present strong synergistic effect. Because of the spread of biofilm forming strains, synergistic antibiotic combinations can be used in DDS to prevent and cure prosthetic joint infection.

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