Follow-up analysis of bacterial species isolated from orthopedic samples

Ph. D. thesis

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List of abbreviations

A = amoxicillin

AMC = amoxicillin/ clavulinic acid

AMK= amikacin

ANOVA= univariate analysis of variance

ASM = American Society for Microbiology

ASTM = American Standards for Test Materials

CE = capillary electrophoresis

CFU = colony-forming unit

CIP = ciprofloxacin

CM = cytoplasmic membrane

CMA = cefamandol

CNS = coagulase-negative *Staphylococcus*

CRO = ceftriaxone

CRP= C-reactive protein

CTX = cefotaxime

CXM = cefuroxime

Da = Dalton

DOX = doxycycline

DSCE = dynamic sieving capillary electrophoresis

E = erythromycin

EDTA = ethylene diaminetetraacetic acid

EMB = eosin methylene blue

ESR = erythrocyte sedimentation rate

G = giga

GE = gentamycin

IPM = imipenem

MATH = microbial adhesion to hydrocarbon

MIC = minimum inhibitory concentration

NE = netilmycin

OD = optical density

OFX = ofloxacin

OMP = outer membrane protein

OX = oxacillin

P = penicillin

PBS = phosphate buffered saline solution

Ps = *Pseudomonas aeruginosa*

R = resistant

S = sensitive

PEG = polyethylene glycol

SAT = salt aggregation test

SDS = sodium dodecyl sulphate

Sta = *Staphylococcus aureus*

THR = total hip replacement

TKR = total knee replacement

TSB = trypticase soy broth

TO = tobramycin

TWANOVA = two-way ANOVA

UHMWPe = ultra heavy molecular weight polyethylen

US = ultrasound

VA = vancomycin

WBC = white blood count

WHO = World Health Organization

1. Introduction

1.1. Clinical background

The issue of wound infection in the clinical practice has been well described since the work of Semmelweis. Wound infection is one of the most dreaded complications of various surgical procedures including the field of orthopedic surgery. This problem affects seriously the patient, the orthopedic surgeon and the attending institution. In the past decades, many studies have investigated the number of infectious cases in the orthopedic practice. Charnley reported an infection rate of 9.5 % following total hip replacement (THR) in the sixties [Charnley, 1964]. Although, according to more recent data, the incidence of infection has been decreasing gradually, it is still about 0.5-2% nowadays [Eftekhar and Tzitzikalakis, 1986, Espehaug *et al*, 1997, Garvin and Hanssen, 1995, Lindwell, 1986]. Since the number of THR increased significantly during this period, the total number of infections also raised [Spangehl *et al,* 1998]. Very important to note that the infections in aged persons have special interest, as the mortality rate of infected THR in elderly patients could reach as high as 8 % [Duggan *et al,* 2001].

Similar results were observed in our department also. In 1996, the Hospital Hygiene Group made a complete fact-finding investigation in the Department of Orthopedics, Faculty of Medicine, University of Pécs. Within that frame the following facts were investigated:

1. The data of wound infection developed following elective orthopedic operation. These were analyzed both qualitatively and quantitatively.
2. The hygienic state of the operating rooms and the ward estimated. The protocols of antibiotic prophylaxis and therapy were analyzed.
3. The team brought up to date the epidemiological attitude of medical doctors, nurses and other workers in the Department.
4. The surveillance system was inserted into the activity of the Department.
5. The group started to scan the financial consequences of wound infections.

Although some parts of the results were published in 1998 [Than et al, 1998], I would like to present a few aspects of this work, which played a role in my choice of both the investigated species of bacteria and antibiotics.

Between the 1st of January of 1996 and 31st of December of 1996, 1656 patients were admitted to our Department. Out of them, 1316 patients underwent WHO coded operations, which was the 79 % of our total patient population. The incident of wound infections is demonstrated in Table I. It is clearly shown that wound infection developed in 20 patients; that is 1.5% of the patients. Analyzing the data in details revealed that infection was observed in 3.4 % of the cases following THR. This was slightly higher than the average according to the international and Hungarian literature [Moser, 1994]. The ratio of wound infection was 4.9 % following spine surgery using metallic implants. In Table II, the types of bacteria isolated from the infected wounds are presented. In Table III and IV, the applied antibiotics and the antibiotic sensitivity of the pathogenic bacteria are demonstrated, respectively. It can be clearly seen that bacteria causing wound infections are the Gram-positive pathogens in most cases (Gram-positive 20, Gram-negative 3 strains). Among the Gram-positive bacteria, *Staphylococcus aureus* and coagulase-negative *Staphylococcus* infections were outstandingly high. In the group of Gram-negative pathogens *Pseudomonas aeruginosa* was found, which species can be hardly eliminated. This is in correlation with the Hungarian [Forgon et al, 1990, Mühsammer and Boda, 1996] and international literature. For example Canner reported that *Staphylococcus epidermidis, Staphylococcus aureus and Escherichia coli* were found in the infected wounds in 75% of the investigated cases, and a single organism was grown on culture in 88% of the patients after infected THR [Canner et al, 1984].

Among the applied antibiotics amoxicillin / clavulinic acid was the most frequently used, but cefuroxime and cefotaxime were also used in our previous investigation. While this is also in correlation with the Hungarian literature [Faluhelyi and Vánkos, 1994, Moser, 1994, Sólyom, 1997], there are authors, however, who suggest clindamycin for antibiotic treatment in orthopedic surgery [Faluhelyi et al, 1998]. Kállay used amikacin in the treatment of infected THR, in which *Acinetobacter* superinfection was found [Kállay and Kullmann, 1994]. The data mentioned above explain as why we investigated these bacterial species and antibiotics in our experimental series.

Table I. Combined data of nosocomial infections in the Orthopedic Department, Faculty of Medicine, University of Pécs from the 1st of January, 1996 to 31st of December, 1996

Type and number of operations		**Number and ratio of postoperative wound infections**	
Hip operations			
With implant	322*	11	3.4 %
Without implant	12	-	-
Knee operations			
With implant	77*	-	-
Without implant	379	-	-
Spine operations			
With implant	61*	3	4.9 %
Without implant	26	-	-
Other type of operations	439	6	1.4 %
Total	1316	20	1.5 %

* In the case of metal work implantation, postoperative follow up of the patients was kept for a year to discover late infections

Table II. List of bacteria causing wound infections regarding to the type of operations

Pathogenic species	**Hip operation (11)**	**Spine operation (3)**	**Others (6)**	**No. of infected operations (20)**
Staphylococcus aureus	5	1	2	8
coagulase-negative *Staphylococcus*	6	0	3	9
Enterococcus	3	0	0	3
Pseudomonas aeruginosa	0	1	0	1
Enterobacter	0	0	1	1
Acinetobacter	0	0	1	1
No. of isolated bacteria	14	2	7	23

Table III. Antibiotic therapy applied in wound infections

Antibiotics	**Hip operations**	**Spine operations**	**Others**
Cefuroxim	1	1	0
Cefotaxim	0	1	1
Amoxicillin / clavulinic acid	8	0	2
Ciprofloxacin	3	1	0
Doxycyclin	3	0	2
Ofloxacin	1	0	0
Total	17	3	5

Table IV. Antibiotic sensitivity of Gram-positive and Gram-negative bacteria isolated from orthopedic wounds: number of sensitive strains/total number of strains.

A. Gram-positive strains

	Enterococcus (3 strains)	Coagulase-negative *Staphylococcus* (9 strains)	*Staphylococcus aureus* (8 strains)
VA	3/3	9/9	8/8
NE	-	7/9	8/8
CTX	-	7/9	8/8
OFX	0/3	7/9	8/8
CMA	0/3	7/9	8/8
TO	0/3	5/9	8/8
CXM	0/3	6/9	8/8
GE	0/3	6/9	8/8
CIP	1/3	7/9	8/8
DOX	3/3	7/9	6/8
AMC	3/3	7/9	8/8
A	1/3	2/9	0/8
E	3/3	4/9	8/8
OX	0/3	7/9	8/8
P	0/3	1/9	0/8

B. Gram-negative strains

	Pseudomonas aeruginosa (1 strain)	*Enterobacter* (1 strain)	*Acinetobacter* (1 strain)
IPM	S	S	S
AMK	S	S	S
NE	S	S	S
CTX	S	S	R
CRO	S	S	R
OFX	R	S	R
CM	R	S	R
TO	S	S	R
CXM	R	S	R
GE	S	S	R
CIP	R	S	R
DOX	R	R	S
AMC	R	R	S
A	R	R	R

The strains are S = sensitive, or R = resistant to the antibiotics determined by filter paper method. Abbreviations of antibiotics are given on page 4 and 5.

It is worth emphasizing that the occurrence of wound infections increased the duration of the hospitalization in average by 8 days, which is an 80 % increase in hospitalization time (Table V). Such interpretation of the data does not give exact results, since the hospitalization in wound infection should be compared to the hospitalization of the same type of operation without infection. But the fact is that after a routine operation of either THR, spine surgery or other operations without complications, the patients are discharged from the hospital latest on the 10^{th} postoperative day.

Table V. Tendency of average nursing time in the case of postoperative wound infection

Following implantation of hip prosthesis	19 days
Following spinal operation	16 days
Following other surgery	20 days
Average number of nursing days	18 days
Average number of nursing days for total number of admitted patients	10 days

Also noteworthy, beside the significant increase of hospitalization, the patients must be treated with relatively expensive antibiotics for long period. These impose significantly greater costs to the hospital. Approximately 2 million HUF was saved in our Department due to the successful infection control in 1996. This lesser expense was related to the cut down only of the antibiotic costs.

According to our own experience and the literature, in case of wound infections, especially if it affects the bones, repeated operations must be performed in many cases [Laky, 1983], which is an extra burden for the patients and further increase in the expenses for the treating institution. I would like to cite the situation in the USA, where approximately 128.000 primary THR were performed in 1993. Out of these, it was between 3500-4000 that became infected and the cost to treat these infections was 150-200 million dollars annually [Sculco, 1993].

Early diagnosis and proper therapy are not easy tasks, thus prevention has special significance especially in elective surgeries. Within the framework of prevention preoperative laboratory examinations, bacteriological cultivation from the pharynx and urine are usually performed to reduce the risk of postoperative wound infections. Furthermore, according to the recommendation of the Antibiotic Committee the patient is given antibiotic prophylaxis for 24 hours in the case of major joint replacement. Even though the strict principles of sterility are followed during surgery and postoperative care, the feared wound infection may develop.

As I mentioned previously, **early diagnosis of wound infection** has a special significance in effective treatment. Nevertheless, establishing the diagnosis of wound infection especially in the early phase is not easy at all. Almost all of the patients has **pain** after surgery, so pain can not play an important role in the diagnosis. Based on only radiological investigations, the diagnosis is difficult to be achieved, because there are no **X-ray signs** of early infection and bone scan shows increased uptake on the affected area especially after bony surgery. **US** can prove **fluid accumulation**, which can be postoperative haematoma as well. **Fever** might develop in these cases, but as it was proven by Than after THR, fever is only a minor predictive factor of wound infection. At the same time the lack of fever does not mean absence of septic complication [Than and Málovics, 2000]. **Blood tests** might help the surgeon to diagnose early wound infection, but the usually investigated parameters (ESR, WBC, CRP) show elevation at the postoperative period in normal cases as well [Sanzén and Carlsson, 1989, Niskanen et al, 1996]. The above mentioned causes played a significant role in **the selection for the topic of my Ph.D. thesis**. That is to establish the normal curve of labor parameters (ESR, WBC, CRP) in the postoperative period, how we can use these parameters for differentiation between infected and non-infected cases after total joint replacement and the tracking microbiological processes in orthopedic wound infections.

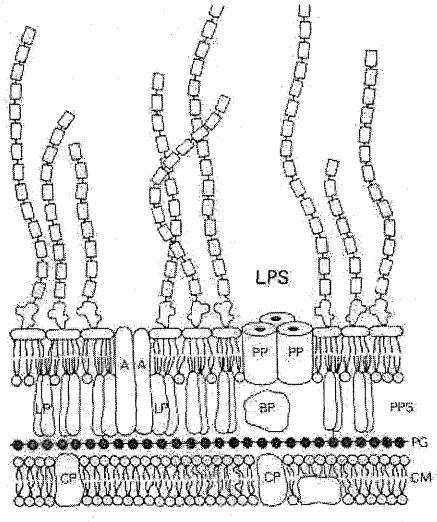
1.2. Adhesion and colonization process

Pathogenesis of bacterial infections is a multi-factorial process. Adhesion of bacteria to the surface of host cells or medical devices is the first, significant step of bacterial infection, followed by colonization, bacterial multiplication, invasion and tissue destruction.

The first, rapid phase of adhesion depends on a variety of non-specific, relatively weak physicochemical forces. Hydrophobic interaction is assumed to be one of the most important factors in rapid attachment process [Christensen et al, 1995]. Beside this electrostatic forces, hydrogen bonds and lectin-like interactions have to be mentioned. Effect of these forces is the immediate, reversible attachment of pathogens to the surface. The first, rapid step of adhesion is followed by the permanent attachment of the loosely held bacteria to the surface. This process is based on specific interactions between

microbial adhesins and receptors of host tissues or implants, and these are usually lectin-saccharide interactions. Outer membrane proteins (OMP) of Gram-negative bacteria are an important group of these adhesins which take part in the attachment process to the host cells (Fig. 1.)

Fig 1. Molecular organization of the outer membrane of gram-negative bacteria. (LPS = lipopolysaccharide; A, OmpA protein, PP = pore protein {matrix porin}, LP = lipoprotein, BP = nutrient-binding protein, PPS = periplasmic space, PG = peptidoglycan, CP = carrier protein, CM = cytoplasmic membrane.) [Jawetz, 1984]



1.3. Surface hydrophobicity of bacterial cells

Studies on bacterial cell-surface characteristics such as charge and hydrophobic properties provided valuable insights in interaction of bacteria with host cell membranes [Smyth *et al,* 1978]. Hydrophobic effect has been known already for decades. First it was considered as a non-specific effect, so the molecular background of this physicochemical force had not been studied in details for a long time. It is already clear, that several bacterial and fungal pathogens use primarily the hydrophobic effect to colonize host tissues. Microorganisms adhere to the host cells or to the surface of medical appliances with the hydrophobic effect, if the surface presents sufficiently high densities of apolar

areas [Doyle, 2000]. Surface of the bacterial pathogens possesses usually hydrophobic as well as hydrophilic components. Several molecules contribute to the hydrophobic character of bacterial cells. Some of them are bounded covalently to cell wall (e.g. Staphylococcal [Meyer and Gatermann, 1994] or Streptococcal proteins [Tylewska *et al,* 1994], or to cytoplasmic membrane (e.g. lipoteichoic acid in *Streptococcus pyogenes* [Hasty *et al,* 1992]). Others are anchored in the outer membrane (e.g. fimbrial proteins [Gonzales *et al,* 1988]), or they can be secreted biomolecules (e.g. amphyphilic polysaccharide of *Acinetobacter calcoaceticus* [Rosenberg *et al,* 1983]). Csh A (259 kDa surface protein) serves as adhesin on the surface of *Streptococcus gordonii*, and gives hydrophobic character for the bacteria [McNab *et al,* 1999]. In *Streptococcus sanguis* a similar surface protein confers hydrophobicity to the cell, and it has an important role in the attachment process to the hydroxylapatite [Nesbitt *et al,* 1982].

1.3.1. Methods for determination of cell-surface hydrophobicity

The role of non-specific hydrophobic interactions in bacterial adherence has led to the development of a wide variety of investigative methods.

Some of them have become standard, such as the **microbial adhesion to hydrocarbon (MATH).** The original MATH method was described by Rosenberg *et al* (1983) and modified by Geertsema *et al* (1993). In this test the aqueous suspension of microorganisms is mixed with hydrocarbons (hexadecane, toluene or xylene), and the hydrophobic cells tend to bind more to the hydrocarbons. This is the most convenient assay for determining the hydrophobic surface characteristics.

Because of its easy performance, the **salt aggregation test (SAT)** is the most commonly employed method, described by Lindalh *et al* [1981]. This technique is based on the premise, that increasingly hydrophobic bacteria will aggregate at correspondingly lower ammonium-sulfate concentrations.

Beside these methods, many others are employed in the practice: the **contact angle method** is probably the most definitive way to determine cell-surface hydrophobicity: one drop of a solvent (water, buffer) is placed on a smooth lawn of dried cells. Dissipation of the droplet depends on the hydrophobic properties of the bacterial surface [Van der Mei *et al*, 1998]. In the **hydrophobic interaction chromatography**

hydrophobic cells tend to adhere more to the octyl-Sepharose column, and can be eluted only with distilled water, chaotropes or low temperature [Smyth *et al,* 1978]. The **two-phase partitioning** technique is based on the fact, that hydrophobic organisms tend to bind strongly to polyethylene glycol (PEG) phase, while hydrophilic bacteria tend to partition into the dextran phase [Pedersen *et al*, 1998]. Adherence to different plastic or glass material e.g. in the **rolling droplet assay** can be used to determine relative cell-surface hydrophobicities [Doyle, 2000].

1.3.2. Hydrophobicity and virulence

The hydrophobic character of bacteria is associated with its virulence and infection. In literature there are several examples on correlation between cell-surface hydrophobicity and infection: *Yersinia enterocolitica* has a surface protein (coded by a plasmid) that possess hydrophobicity to bacteria. The infective, hydrophobic strains are carrying the plasmid, while the plasmid-deficient mutants have hydrophilic surface and show no pathogenicity [Lachica and Zink, 1984]. The enterophatogenic *Escherichia coli* strains causative agent of diarrhoea exhibit hydrophobicity. The P-fimbriated *E. coli* strains causing pyelonephritis were characterized by hydrophobic cell-surface properties [Jacobson *et al,* 1989]. Adhesins of *S. aureus,* which are various extracellular matrix binding proteins capable of binding collagen, fibronectin, laminin, etc., have active sites, which contain primarily hydrophobic amino acids [Foster and Höök, 1998]. Bacterial adhesion increases with higher hydrophobicity and decreases with lower hydrophobicity [Hostacka and Karelova, 1997]. Bacterial strains having more hydrophilic surface show decreased adhesion to living tissues and to medical implants as well.

1.4. Outer membrane proteins (OMP) of Gram-negative bacteria

The cell envelope of Gram-negative bacteria differs markedly from the Gram-positives, and is composed of three distinct layers. The inner (or cytoplasmic) membrane is separated from the outer membrane by a monolayer of peptidoglycan. The two membranes differ both in function and composition. The outer membrane contains a few proteins in large quantity, the so-called "major" outer membrane proteins (OMP)

[Nishimura *et al*, 1996, Vaara and Nikaido, 1984]. Beside that phospholipids, and lipopolysaccharides are the components of this membrane.

It was already proven, that OMPs are responsible for several bacterial functions. They are important pathogenic factors, they participate in the attaching process to the host cells. As surface antigens they play an important role in the immunological response of the host [Datta *et al*, 1977, Yu and Mizushima, 1982]. From functional point of view, the best-known OMPs are the porins, which produce pores and promote the transport of different substances. One group of porins produces relatively nonspecific channels that allow the passage of small hydrophilic molecules across the outer membrane. OMPs of *P. aeruginosa* differ from the porins of enteric bacteria. *Pseudomonas* outer membrane proteins form significantly larger pores than enteric porins and allow the diffusion of larger hydrophilic molecules (up to 6000 Da) [Hancock and Nikaido, 1978]. However, these pores are more often in a closed state than those of *Enterobacteriaceae* [Lugtenberg, 1986]. Consequently, the total area of pores available for diffusion is considerably reduced in *P. aeruginosa*. This low outer membrane permeability explains the high natural resistance of *P. aeruginosa* to hydrophilic antibiotics [Hancock *et al*, 1982; Hostacka and Karelova, 1997b]. Other group of porins is involved in specific diffusion processes. For example LamB protein in the passage of maltose and maltodextrins, Tsx involved in nucleoside transport, FhuA protein in ferrichrome-, BtuB in vitamin B12 and FepA protein in Fe^{3+}-enterochelin transport. Furthermore these proteins serve as receptors for various bacteriophages (T6, T5, T1, λ) and colicins (colicin B, D, E, K) [Vaara and Nikaido, 1984].

1.5. Colonization of orthopedic prosthetic devices

In the recent years advances in medical implants (catheters, prostheses and cardiac valves) have made great strides in medicine [Jouenne *et al,* 1994]. But this progress has been accompanied by the emergence of implant-associated infection [Darouiche *et al,* 1994]. This phenomenon has great significance in orthopedic surgery, because of the increasing number of implantation of joint (hip and knee) prostheses and other implants.

Like colonization of living tissues, the bacterial adhesion and colonization of non-living medical devices is a multiphasic process (See Ch. 1.2.), but most of the

microorganisms tend to colonize non-living things rather than host tissues. Although the majority of these infections are caused by coagulase-negative *Staphylococci,* but the full range of pathogenic and saprophytic microbes can be responsible for implant-associated infections.

After the colonization process, bacteria are able to form **biofilm** on the surface of artificial medical devices, which might be demonstrated by direct electron microscopic studies. The cells bind firmly to the surface of the device by producing exopolisaccharide glycocalix polymers [Fletcher, 1977], forming a matrix inside, in which microcolonies develop [Anwar *et al,* 1990; Holmes, 1990]. It has been established (by scanning confocal laser microscopic observations) that biofilms produced by all bacterial species have similar structures [Anwar *et al* 1990]. Biochemically it consists of uronic acid, mono- or disaccharides, such as mannose, galactose, fucose and aminosugars. The presence of biofilms may explain the persistent nature of biomaterial centered infections, its persistent nature to antibiotic treatment [Hoyle et al, 1990; Anwar *et al,* 1990], the ability to avoid attack by host defenses and the frequent requirement for prosthesis removal. The social and economic benefits from the efficient control of biofilms and the prevention of chronic infections would be numerous.

There are several **experimental protocols** for studying the colonization of bacteria on medical devices [Christensen *et al,* 1995]. The classic approach to quantify attached microorganisms is the direct observation (by optical microscope, or by scanning electron microscope). Limitations of these methods are the tedium of counting large numbers of microscopic fields, and the individual bacteria cannot be reliably counted. It has been applied for example, to determine the number of attached *Pseudomonas cells* on polystyrene Petri dishes [Feltcher, 1977]. Application of radiolabeled bacteria is the most sensitive and versatile assay, but requires specialized instruments and it is expensive [Vaudaux *et al,* 1993]. Staining of bacterial biofilms has two types: *test tube method* is a qualitative method proving the macroscopic presence or absence of biofilm [Christensen *et al,* 1982], while the *microtiter plate method* uses an automatic spectrophotometer to read the optical density of the stained biofilms [Fletcher, 1976]. Biological assays are sensitive, easily performed, and applicable for a wide variety of complex objects. Disadvantages are the requirement of specialized equipment and reagents, and the

necessity of a standardization step. Either ATP production [Kristinsson, 1989] or cell-associated urease production can be detected. Counting of living detached organisms includes detachment of surface-associated microorganisms and counting the number of colony-forming units. There are two basic approaches: the roll technique and sonication. The *roll technique* is simple and requires little in equipment and reagents. But it can be used only in small-gage cylindrical objects, and it does not measure intraluminar colonization [Maki *et al*, 1977]. *Sonication* was applied in our experimental series, detailed methodical discussion, advantages and disadvantages of the technique are described in Chapter 3.6.

1.6. Effect of antibiotics on adhesive ability

Antibiotic treatment is able to induce several morphological and metabolical changes in bacterial strains. Alterations in bacterial adherence, in the ultrastructure of cells, in the protein and/or DNA biosynthesis, and in production of some enzymes and bacterial virulence factors have been already observed [Hostacka and Karelova, 1997b, 1997c,1997d, Manek *et al*, 1986].

Antimicrobials at supra-inhibitory and also at sub-inhibitory concentrations can modify the physicochemical characteristics of the cell-surface. Hostacka and Karelova [1997a] documented, that the cell-surface **hydrophobicity** of *Shigella dysenteriae* type 1 strain was decreased after the exposure to different aminoglycosides. Similar results were published by Braga and Reggio [1995], who found that brodimoprim decreased effectively the surface hydrophobicity of *S. aureus* strains. Significance of these results, that bacterial adhesion increases with higher hydrophobicity and decreases with lower hydrophobicity. Bacterial strains having more hydrophilic surface show decreased adhesion to living tissues and to medical implants as well.

More papers discussed in details the changes of **OMP profiles** during antibiotic effect: Hostacka and Karelova (1997b) found, that norfloxacin was able to induce an overproduction of the 41kDa protein, and reduction of 23 and 45 kDa proteins in the patterns of *Pseudomonas aeruginosa* strains. Giordano *et al* [1993] reported, that the use of quinolones caused an evident increase in the amount of 38 and 41 kDa proteins in the OMP profiles of *Pseudomonas aeruginosa* strains. Kustos *et al* [1998] discussed the

modifying effect of meropenem on the OMP composition of different enterobacterial strains. Changes in OMP profiles, and thus in the surface composition of the bacterial pathogens may cause dramatic alterations in the adhesive ability. These changes might result in altered virulence and pathogenicity of the bacterial strains, thus detection of these modifications has medical importance.

2. Aim of the study

Aims of this study were as follows:

1. To establish the normal range of values in case of three laboratory parameters (ESR, WBC, CRP) pre- and postoperatively after total joint replacement in non-infected cases. These laboratory parameters are often used to help the surgeon to set up diagnosis of infected total joint replacement.

2. To isolate pathogenic bacteria from the infected wounds of orthopedic patients, and identify them on the basis of standard microbiological methods (see Ch. 3.2.).

3. To analyze the antibiotic sensitivity of the isolated *S. aureus*, coagulase-negative *Staphylococcus* and *Pseudomonas aeruginosa* strains, determine the minimum inhibitory concentrations (MIC) of four antibiotics on these strains (cefuroxime, cefotaxime, amoxicillin + clavulinic acid and amikacin). These four antibiotics were chosen, because they are applied most often in our department in prophylaxis and treatment of patients.

4. To determine the cell-surface hydrophobicity of the isolated strains, and compare their surface characteristics to three Hungarian standard bacterial strains. Hydrophobicity of the cells was determined by two different methods: salt aggregation test (SAT) and microbial adhesion to hydrocarbons (MATH). Our aim was to compare the results obtained by the two techniques.

5. To examine the effect of the four antibiotics mentioned above at sub-inhibitory and supra-inhibitory concentrations on cell-surface hydrophobicity.

6. To analyze the outer membrane protein composition of the *Pseudomonas* strains isolated from orthopedic wounds by capillary electrophoresis (which is a new technique applied first in our University for the analysis of bacterial proteins). To compare their OMP profiles with the Hungarian standard *P. aeruginosa NIH Hungary 170000,* and determine the modifying effect of the antibiotics at supra-inhibitory concentrations.

7. To analyze the ability of adhesion of the bacterial strains to orthopedic implants by the sonication method. To examine whether the antibiotics can modify the attachment process at sub-inhibitory or supra-inhibitory concentrations.

8. To find correlations between cell-surface hydrophobicity, OMP composition and the adhesive ability of the bacterial strains isolated from clinical sources.

9. To compile recommendations for the clinical practice on the base of our experimental results.

3. Materials and methods

3.1. Laboratory parameters in patients following non-infected total joint replacement

In the first part of our work 50 patients were selected randomly, for investigation. These patients underwent total joint replacement (THR, TKR) in the Department of Orthopedics, University of Pécs. The aim of this study was to determine how the commonly used laboratory parameters changed in patients following non-infected total joint replacement.

The next laboratory parameters were investigated by the Central Clinical Laboratory in Pécs University as a routine method:

1. erythrocyte sedimentation rate (ESR)
2. number of white blood cells (WBC)
3. C-reactive protein (CRP)

The criteria for patient selection were:

1. None of the patients suffered from any kind of already known disease, which could influence the above mentioned laboratory parameters, like diseases effecting the immune system, gout, diabetes mellitus etc.
2. The selected patients had no previous total joint replacement, or any kind of major orthopedic operations with or without metalwork implantation.
3. On admission the ESR was in the normal range (ESR< 20 mm/h).
4. The patients had negative bacteriological culture from the pharynx and urine.

During our work the ESR, WBC and the CRP levels of the above mentioned patients were measured preoperatively and on the 1^{st}, 3^{rd} and 10^{th} postoperative days. Ultimately, the data of those patients were excluded, who developed wound infection within 3 months after the surgery. One patient, an 80 year old lady was excluded, who had cemented type THR. Wound infection developed in 1 month postoperatively, and this infection was indicated by our laboratory data.

The data of 49 patients were analyzed. The mean age of our patient population was 53.9 years (21-73 years).

The diagnoses of the patients are shown in Table VI.

Table VI. Distribution of the patients according to their diagnosis

Diagnosis	Number of patients
Avascular femoral head necrosis	7
Osteoarthritis of the hip	37
Osteoarthritis of the knee	5

THR was performed in 44 cases, while TKR in 5 cases.

For analysis of our results non-parametric procedures were applied (Wilcoxon serial test, Siegel-Tukey test).

3.2 Patients with infected wounds and bacterial strains

In the second part of our work patients having wound infections following orthopedic surgeries had been selected randomly for our study. Surgeries were performed in 2000/2001 in the Department of Orthopedics, Faculty Medicine, University of Pécs. Seven patients aging between 14 and 87 years (all of them females) were investigated. Acute infection was observed in four patients and chronic one in three cases. 13 bacterial strains (five *S. aureus,* three coagulase-negative *Staphylococci*, and five *P. aeruginosa*) isolated from the wounds of the patients were analyzed in our experimental series. The strains were identified by standard bacteriological methods [Kiska, 1999 & Kloos, 1999]. The most important data of patients and bacterial strains are summarized in Table VII. Beside the clinical species, three Hungarian standard bacterial strains were also examined as controls: *Staphylococcus aureus* NIH Hungary 118003, *S. saprophyticus* NIH Hungary 120008 and *Pseudomonas aeruginosa* NIH Hungary 170000.

Table VII. Bacterial strains investigated in the experiments were isolated from wounds of orthopedic patients. Age, sex, diagnosis, and the type of surgery applied on these patients are summarized in the Table.

Bacterial strain	**Type of bacteria**	**Age**	**Sex**	**Diagnosis**	**Type of operation**
KT 1	*S. aureus*	14	Female	Scoliosis	Postero-lateral fusion with Cottrell-Dubousset instrumentation
KT 2	*P. aeruginosa*	16	Female	Scoliosis	Postero-lateral fusion with Cottrell-Dubousset instrumentation
KT 3	*S. aureus*	16	Female	Scoliosis	Postero-lateral fusion with Cottrell-Dubousset instrumentation
KT 7	*P. aeruginosa*	38	Female	Spondylolysthesis L V. After L. IV.- S. I. fusion	Metal implant removal
KT 23	*S. aureus*	54	Female	Osteoarthritis of the knee Arthrodesis of the knee joint	Fixature extern. removal
KT 24	Coagulase-negative *Staphylococcus*	54	Female	Osteoarthritis of the knee Arthrodesis of the knee joint	Fixature extern. removal

KT 25 Sta.	*S. aureus*	87	Female	Osteoarthritis of the hip Infected THR	Prosthesis removal from the hip joint
KT 25 Ps.	*P. aeruginosa*	87	Female	Osteoarthritis of the hip Infected THR	Prosthesis removal from the hip joint
KT 26	Coagulase-negative *Staphylococcus*	87	Female	Osteoarthritis of the hip Infected THR	Prosthesis removal from the hip joint
KT 27	*S. aureus*	54	Female	Osteoarthritis of the knee Arthrodesis of the knee joint	Fixature extern. removal
KT 28	*P. aeruginosa*	87	Female	Osteoarthritis of the hip Infected THR	Prosthesis removal from the hip joint
KT 278	Coagulase-negative *Staphylococcus*	58	Female	Avascular femoral head necrosis	THR
KT 39	*P. aeruginosa*	74	Female	Tumor of the left proximal femur	Resection of the tumor and THR

3.3. Identification of bacterial strains isolated from orthopedic wounds

Bacterial strains were identified by standard microbiological methods [Kiska, 1999, Kloos, 1999].

3.3.1. Gram staining

Gram staining method (named after the Danish bacteriologist devised it in 1884, Hans Christian Gram) is one of the most important staining techniques in microbiology. It is a four part procedure. The specimen has to be mounted and fixed on a slide before staining.

In the first step slides were covered with crystal violet for 1 minute. Then slides were washed with running water and in the next phase they were covered by iodine solution (potassium iodide) for 1 minute as well. After rinsing with water addition of the decolourizer (alcohol) was performed. It had to be added drop by drop until the blue-violet color is no longer emitted from the specimen. The cell walls of the Gram-negative bacteria have a very low affinity for the violet stain, which is rinsed out by the alcohol. The final step involves applying the counter stain (basic fuchsin) for 2 minutes. Gram-positive cells will incorporate little or no counter stain, and will remain blue-violet. Gram-negatives however take on red color and easily distinguishable from Gram-positives.

3.3.2. Cultivation

Cultivation of bacterial strains were performed on nutrient agar, blood agar and eosin methylene blue (EMB) agar.

Nutrient agar contains ca. 2 % of agar and support the growth of many organisms which are not nutritionally fastidious (e.g. *Staphylococcus* or enterics).

The specimen were plated to **blood agar** as well, because it supports the growth of most medically important organisms. It consists of basal medium, 2 % of agar, and enriched with 5 % defibrinated sheep blood. Haemolysins produced by some species cause the lysis of the red blood cells. Type of the haemolysis can be used in preliminary identification.

EMB agar contains methylene blue and eosin dyes to inhibit the growth of Gram-positive bacteria. It is a differential medium, it differentiates on the basis of lactose fermentation. Small amount of acid production results in a pink colored growth, while large amounts of acid cause a precipitation on the colonies resulting in a characteristic greenish, metallic sheen.

3.3.3. Biochemical tests

3.3.3.1. Coagulase test

Coagulase is a protein of unknown chemical composition having a prothrombin-like activity capable of converting fibrinogen into fibrin, which results in the formation of a visible clot in a suitable test system. Function of coagulase *in vivo* is producing of a fibrin barrier at the site of *Staphylococcal* infection, and localization of the organism in abscesses. In laboratory, the coagulase test is used to differentiate *S. aureus* from the other *Staphylocci.*

0.5 ml of a 24 h pure broth culture of the organism was added to 0.5 ml plasma. It was mixed gently and placed to 37°C. The reaction was considered positive, if any degree of clothing was visible. Tubes were observed after 4 and 24 hours.

3.3.3.2. Oxidase test

Oxidase test is the most helpful method in identifying of colonies suspected of belonging to genera such as *Pseudomonas, Aeromonas,* etc. These strains give positive oxidase reaction.

The cytochrome oxidase test utilizes certain reagent dyes, such as p-phenylenediamine dihydrochloride, that substitute for oxygen as artificial electron acceptors. In reduced state the dye is colorless, however in the presence of cytochrome oxidase and atmospheric oxygen it is oxidized forming indophenol blue.

In this method paper strip was applied: a few drops of the reagent was added to a filter paper strip. A loopful of suspected colony was smeared into the reagent zone of the filter paper. Bacterial colony having oxidase activity develop a deep blue color at the inoculation site within 10 seconds.

3.4. Antibiotics and determination of the minimum inhibitory concentrations

Four antibiotics – applied most often in our Department for prophylaxis and treatment of patients – were used: Zinacef (Eli Lilly Italia, S.P.A. Sesto Fiorentino, Florence, Italy), containing cefuroxime; Claforan (Hoechst Marion Roussel, France), containing cefotaxime; Aktil (Richter, Hungary) containing amoxicillin and clavulinic acid with a ratio 2 to 1; and Amikin (Bristol-Myers Squibb, USA) containing amikacin. The antibiotics have different pharmacodynamics: cefuroxime and cefotaxime are cephalosporins and able to induce changes in the cell-membrane, the combination of amoxicillin and clavulinic acid has the same effect, and beside that it can inhibit β-lactamases, while the effect of amikacin is the inhibition of protein synthesis.

The minimum inhibitory concentration (MIC) values were determined by the tube dilution method. Each tube contained 1 ml broth, and a twofold dilution was performed with the antibiotic stock solution (800 μg/mL) [Jorgensen, 1999]. Then 10-10 μL-s of the bacterial cultures (absorbance at 600 nm was ca. 0.2) were added to each tube, and incubated for 24 h at 37°C. The lowest concentrations of the antibiotics inhibiting the growth of the bacterial strains after 24 h incubation were regarded as MIC.

3.5. Determination of bacterial cell-surface hydrophobicity

3.5.1. Salt aggregation test (SAT)

Hydrophobicity of bacterial cell surface was determined by the method of Lindahl et al (1981). One drop (10 μL) of control or antibiotic treated bacterial suspension (5 x 10^9 bacteria / mL) in 0.04 M sodium phosphate buffer (pH = 6.8) was mixed with 10 μL of increasing concentrations of ammonium sulphate solution. The final concentration of $(NH_4)_2SO_4$ changed between 0.05-4.00 M / L. The mixture was gently shaken on a glass slide for 1 minute. The lowest concentration of ammonium sulphate at which aggregation of bacteria occurred was regarded to be characteristic for the cell-surface hydrophobicity of the bacterial strain.

In our study effects of antibiotics were analysed on the way, that four different type of antibiotics in 0.5 MIC and 2 MIC concentration were used for 60 minutes for incubation before the centrifugation.

3.5.2. Microbial adhesion to hydrocarbons (MATH)

This is the most convenient assay for determining cell-surface hydrophobicity. This method was described first by Rosenberg *et al* (1983) and modified by Geertsema *et al.* in 1993. Three different hydrophobic solvents were applied in our experiments: toluene, xylene and hexadecane. Bacteria were cultivated in 100 ml of nutrient broth at 37°C with shaking (100 rpm), collected by centrifugation (4500g, 15 min, 4°C), and washed with physiological saline. Cell pellets were resuspended in physiological saline to obtain optical density 0.4 - 0.6 at 600 nm. Then 3 ml of the hydrophobic solvent was added to 3 ml of the bacterial suspension, and vortexed vigorously for 1 minute. After it, the mixture was kept at standstill for five minutes, till the hydrophobic solvent and the aqueous phase separated. The aqueous phase was removed and the absorbance was measured at 600 nm. The percent of hydrophobicity was determined on the basis of the following equation:

$$\% \text{ hydrophobicity} = \frac{OD_{before} - OD_{after}}{OD_{before}} \times 100$$

In our study effects of antibiotics were analysed on the way, that four different type of antibiotics in 0.5 x MIC and 2 x MIC concentration were used for 60 minutes for incubation before the centrifugation

3.6. Studying microbial colonization of orthopedic medical devices

There are several methods for studying bacterial adherence to artificial surfaces. In our experimental series a sonication method was applied. This is one of the most efficient methods in enumerating surface microorganisms detached to a medical implant and count the colony-forming units recovered from the procedure. Results are quantitative, direct, easily standardized and do not involve special technology or materials. It can be performed on a variety of subjects with complex shapes. It has also drawbacks, because the detachment process may not be complex or maybe harmful for the cells. Despite these drawbacks this technique has wide applicability in the examination of medical devices [Christensen *et al*, 1995].

3.6.1. Orthopedic implants

Acetabular polymer technology has remained largely unchanged for twenty years. Over the years, polyethylene has proved to be a safe implant material, presenting the lowest risk of complication. For our research work a Johnson & Johnson Ultima cemented type acetabular cup was used. The material of this implant is UHMWPe. The quality of "medical grade" UHMWPe is governed by ASTM F/648 and ISO 5834. The acetabular cup was cut for 10 almost equal pieces with an oscillating saw. It was cleaned mechanically and sterilized in gas before every experiment.

3.6.2. Quantitative bacterial adherence

Adhesive ability of nine bacterial strains to the surface of polyethylene acetabular cup was analyzed. Adherence of the three Hungarian standard strains (*S. aureus* NIH Hungary 118003, *S. saprophyticus* NIH Hungary 120008 and *P. aeruginosa* NIH Hungary 170000). Beside them two CNS strains (KT 24 and KT 278), two *S. aureus* (KT 1 and KT 25 Sta.), and two *P. aeruginosa* (KT 2 and KT 25 Ps.) isolated from the wounds of orthopedic patients were examined.

Bacterial strains were plated on blood agar and incubated for 18 h at 37°C. Bacteria were inoculated to 30 ml of TSB with 0.25 % dextrose and incubated for 24 h on 37°C with shaking (100 rpm). Bacteria were collected by centrifugation (4500 g, 15 min, 4°C), and washed two times with phosphate buffered saline solution (PBS) [Siverhus et al, 1990]. Optical density of the suspension was corrected spectrophotometrically to 0.4 at 600 nm. Graft specimens were placed in 30 ml of the bacterial suspensions and incubated for 2 h at 37°C. Ideal incubation period is usually a source of confusion. The term 'adherence' is offered to use only when referring exclusively to immediate attachment (incubation periods of 2-180 min). In the case of longer incubation periods the terms 'microcolony formation', 'biofilm formation', 'slime production', etc. should be used [Christensen *et al*, 1995].

After incubation grafts were washed three times with 25 ml of PBS solution and then placed to 25 ml of PBS with 0.25 % dextrose and oscillated for 1 minute at 125 W (MSE machine) [Chang and Merritt, 1991]. One ml of the sonicated effluent was

removed and serial dilution was performed with physiological saline. 10 µl of each tube was placed to nutrient agar and incubated for 24 h at 37°C. Bacterial adherence was calculated from the number of the colony-forming units (CFUs) and expressed as the number of CFUs per square centimeters of the graft material.

3.7. Analysis of outer membrane proteins in *Pseudomonas aeruginosa* strains

3.7.1. Preparation of outer membrane proteins

Bacteria were cultivated in 2000 ml of a medium, which contained bacteriological peptone (1.667 %), Na_2HPO_4 (0.11 %), glucose (0.389 %), beef extract (0.195 %), and $MgCl_2$ (0.023%) pH=7.2. Bacteria were collected by centrifugation (5000 g, 15 min, 4°C) and then washed with physiological saline.

Bacterial cells were plasmolysed by resuspending the cell pellet in 25 ml of 0.75M sucrose / 10 mM Tris, pH=7.5 buffer. Peptidoglycan layer of the *Pseudomonas* cell wall was digested by lysozyme treatment (2.5 mg lysozyme for 2 minutes at 4 °C). Then, 50 ml of cold 1.5 mM EDTA, pH=7.5 was added slowly (within 10 minutes), which diluted the suspension, and simultaneously converted the originally rod-shaped bacteria to spheroplasts. Lysis of spheroplasts was performed by sonication (MSE type, 400 W), in 35 ml volumes for 2 minutes. Unbroken cells were removed by centrifugation (5000 g, 15 minutes, 4 °C). The membrane fractions of the lysates were collected by ultracentrifugation (100.000 g, 2 x 1 h, 4°C). The inner and outer membranes were separated by sucrose gradient ultracentrifugation. The pellets were resuspended in 3 ml of 25% (w/w) sucrose in 5 mM EDTA, pH=7.5, and loaded on the top of a sucrose step gradient of 30-55% (w/w) in 5 mM EDTA, pH=7.5. The gradients were centrifuged at 100.000 g in a SW 28 rotor of a Spinco ultracentrifuge (Beckman, Spinco Division, Palo Alto, CA, USA) for 21 h at 4°C. After centrifugation outer membrane (OM) and cytoplasmic membrane (CM) could be seen as separate turbid bands. They were collected in several fractions. The OM fractions were washed by deionized water (Milli-Q, Millipore, Bedford, MA, USA), and centrifuged (100.000 g, 2 h, 4°C). Pellets were resuspended in 500 µl of sample buffer and 500 µl of deionized water, and stored at -20°C.

3.7.2. Capillary electrophoresis

In the capillary electrophoretic measurements the dynamic sieving capillary electrophoretic technique (DSCE) was applied [Kustos *et al*, 1998a, 1998b]. This method ensured the separation of outer membrane proteins on the basis of their molecular weights. Sieving effect was provided by the hydrophil polymer of the commercially available CE-SDS Protein Run Buffer of BioRad (BioRad, Richmond, CA, USA).

Measurements were performed by the BioFocus 3000 System (BioRad Laboratories, Hercules, CA, USA), in a 24 cm long, 50 μm internal diameter, uncoated, fused silica capillary. Separation was achieved at constant voltage (15 kV), the current limit was 50 μA. Temperature of the cartridge and the carrousels were stabilized at 20°C. Samples were injected hydrodinamically by 50 psi x sec pressure. Capillary was washed between each run by 0.1 M NaOH, 0.1 M NaCl and deionized water. UV detection was performed at 220, 254 and 280 nm. Data were stored in personal computer.

In each run an internal standard (benzoic acid) was applied, which appeared before the bacterial proteins. For evaluation of the molecular weights of proteins the low molecular weight calibration kit of Pharmacia (Pharmacia Biotech, Uppsala, Sweden) was applied (α-lactalbumin, 14.4 kDa; trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; albumin 67 kDa; phosphorylase b, 94 kDa). Relative percentage of the peak areas was determined by the Biofocus Integrator program.

3.8. Statistical analysis

Statistical evaluation of the data obtained in our experiments were performed by univariate and two-way ANOVA tests (analysis of variance). Statistical significance was accepted when p-values (significance levels) were less than 0,001 ($p<0,001$). Empirical F-values were also calculated for determining the factors causing significant changes in hydrophobicity and adhesive ability (the higher the empirical F-value, the stronger the relationship between the factor examined and the cell-surface hydrophobicity or adhesive ability). Adjusted R-squared values were determined to show the responsibility of one or more factors in the values obtained.

For the analysis of changes in individual data one sample one sided Z-test was applied with the next conditions:

a. sample size is greater than 30
b. distribution is normal
c. standard deviation is not higher than the control value (so the sampling error is maximum 100 %)

4. Results

4.1. Changes in laboratory parameters in patients following non-infected total joint replacement

Our results are summarized below:

Tendency of ESR: Normal range: 1-20 mm/h

Preoperative:	*10,4 mm/h*	*(2-20 mm/h)*
1st postoperative day:	*26,3 mm/h*	*(6-62 mm/h)*
3rd postoperative day:	*49,3 mm/h*	*(14-115 mm/h)*
10th postoperative day:	*53,8 mm/h*	*(12-98 mm/h)*

It can be seen clearly, that ESR is meaningfully increased even in "normal", non-infected cases and it was still slowly increasing on the 10th postoperative day.

WBC values: Normal range: 4-10,0 G/l

Preoperative:	*6,5 G/l*	*(4,0-11,8 G/l)*
1st postoperative day:	*8,8 G/l*	*(4,5-20,2 G/l)*
3rd postoperative day:	*7,8 G/l*	*(4,3-15,7 G/l)*
10th postoperative day:	*7,3 G/l*	*(3,9-10,5 G/l)*

In non-infected cases, the WBC level shows a mild increase on the 1st postoperative day, than it started to decrease.

CRP values: Normal range: < 10 mg/l

Preoperative:	*4,8 mg/l*	*(0-39,5 mg/l)*
1st postoperative day:	*45,9 mg/l*	*(2,4-117 mg/l)*
3rd postoperative day:	*61,1 mg/l*	*(2,0-143 mg/l)*
10th postoperative day:	*31,4 mg/l*	*(1,0-109 mg/l)*

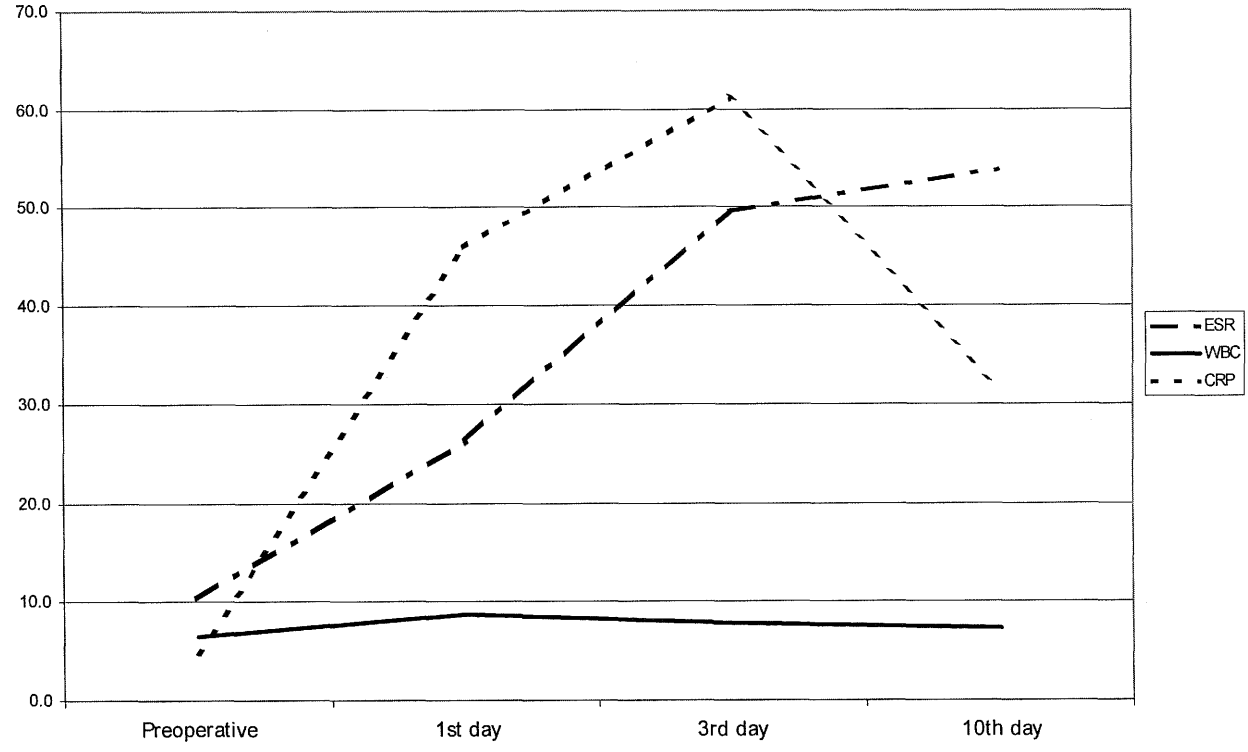
It can be seen that, there is a significant increase in the CRP values immediately after the surgery, but it tends to return to the normal level on the 10th postoperative day.

There was one patient among the 50 patients, an 80 years old female, as it was mentioned in Chapter 3.1, who developed infection after THR within a month. Her CRP value did not decrease after the 3rd postoperative day, in spite we detected an increase in her CRP level. According to our opinion, this laboratory parameter among the examined,

is the most sensitive and most useful marker for the surgeon to set up diagnosis in case of wound infection.

The changes of the three laboratory parameters are summarized in Fig. 2.

Fig. 2. Preoperative and postoperative values of ESR, WBC and CRP in orthopedic patients with non-infected total joint replacement.



4.2. Bacterial strains isolated from orthopedic patients with infected wounds

Thirteen bacterial strains were isolated from the wound of seven orthopedic patients, who had been operated on in our department in 2000/2001. All of them had postoperative infections. On the basis of standard microbiological methods we could identify five *S. aureus,* three coagulase-negative *Staphylococci*, and five *P. aeruginosa* strains. (Further details in Ch. 3.2.)

4.3. Determination of minimal inhibitory concentration of antibiotics

The minimum inhibitory concentration (MIC) values of the four selected antibiotics – determined by the two-fold dilution method - on the three standard strains and on the bacteria isolated from orthopedic wounds are summarized in Table VIII. MIC values of cefuroxime in the *Staphylococcus* strains varied between 0.4 and 1.56 μg/mL, while in the *Pseudomonas* strains the levels were above 100 μg/mL. The MIC values of cefotaxime in the *Staphylococci* were between 0.8 and 6.25 μg/mL, and in the *Pseudomonas* strains between 50 and 100 μg/mL. Minimum inhibitory concentration of amikacin varied between 0.4 and 12.5 μg/mL, while amoxicillin + clavulinic acid was able to inhibit the growth of *Staphyloccus* strains between 0.2 and 50 μg/mL, and the growth of *Pseudomonas* strains only in high concentrations (at 200-800 μg/mL).

Table VIII. Minimum inhibitory concentration (MIC) of cefuroxime, cefotaxime, amoxicillin + clavulinic acid and amikacin in the bacterial strains examined.

Strains	**MIC values (μg/ml)**			
	cefuroxime	**cefotaxime**	**amoxicillin + clavulinic acid**	**amikacin**
S. saprophyticus NIH Hungary 120008	1.56	3.125	0.2	0.4
S. aureus NIH Hungary 118003	0.8	0.8	0.2	0.4
P. aeruginosa NIH Hungary 170000	>100	50	400	1.56
Clinical isolates:				
Coagulase-negative *Staphylococcus*	0.4 – 0.8	0.8 – 6.25	0.2	0.4 – 1.56
S. aureus	0.4 - 0.8	0.8 – 3.125	0.2 – 0.4	0.8 – 6.25
P. aeruginosa	100 - >100	50 - >100	200 - 800	1.56 – 12.5

4.4. Determination of hydrophobicity by the salt aggregation test:

Visible cell aggregation of untreated *S. aureus,* coagulase-negative *Staphylococcus,* and *P. aeruginosa* strains occurred between 1.0 and 3.0 molar concentration (M/L) of ammonium-sulphate. We could not detect any significant difference between the hydrophobicity of clinical isolates and the Hungarian standard coagulase-negative *Staphylococcus* (2.5 M/L), *S. aureus* (2.0 M/L) and *P. aeruginosa* (2.0 M/L) strains.

Both the standard strains and the orthopedic isolates were subjected to sub-inhibitory and supra-inhibitory antibiotic treatment with cefuroxime, cefotaxime, amoxicillin + clavulinic acid, and amikacin. Cell-surface hydrophobicity of the control and antibiotic treated coagulase-negative *Staphylococci, S. aureus,* and *P. aeruginosa* strains determined are shown in Figure 3. In each figure we presented the $(NH_4)_2SO_4$ concentration characteristic for the untreated strain, and the changes after 0.5 x MIC and 2 x MIC concentrations of (a) cefuroxime, (b) cefotaxime, (c) amoxicillin + clavulinic acid and (d) amikacin treatment.

Our results showed that the hydrophobicity of cell-surface – studied by the salting out test – was changed in several cases after exposure to different types of antibiotics in the standard strains and in the clinical isolates as well. These alterations mean that visible cell aggregation occurred at higher $(NH_4)_2SO_4$ concentration, so the hydrophobicity was decreased. Changes in surface hydrophobicity were observed more often after treatment with supra-inhibitory concentration of antibiotics.

Figure 3. Concentrations of $(NH_4)_2SO_4$ solutions characteristic to cell surface hydrophobicity of bacteria. In each figure we presented hydrophobicity of coagulase-negative *Staphylococci* in the first, *S. aureus* in the second, and *P. aeruginosa* strains in the third group. Hydrophobicity of the untreated strains and the changes after sub-inhibitory (0.5 x MIC) and supra-inhibitory (2 x MIC) treatment of *(a)* cefuroxime; *(b)* cefotaxime; *(c)* amoxicillin + clavulinic acid and *(d)* amikacin are shown in the figures.

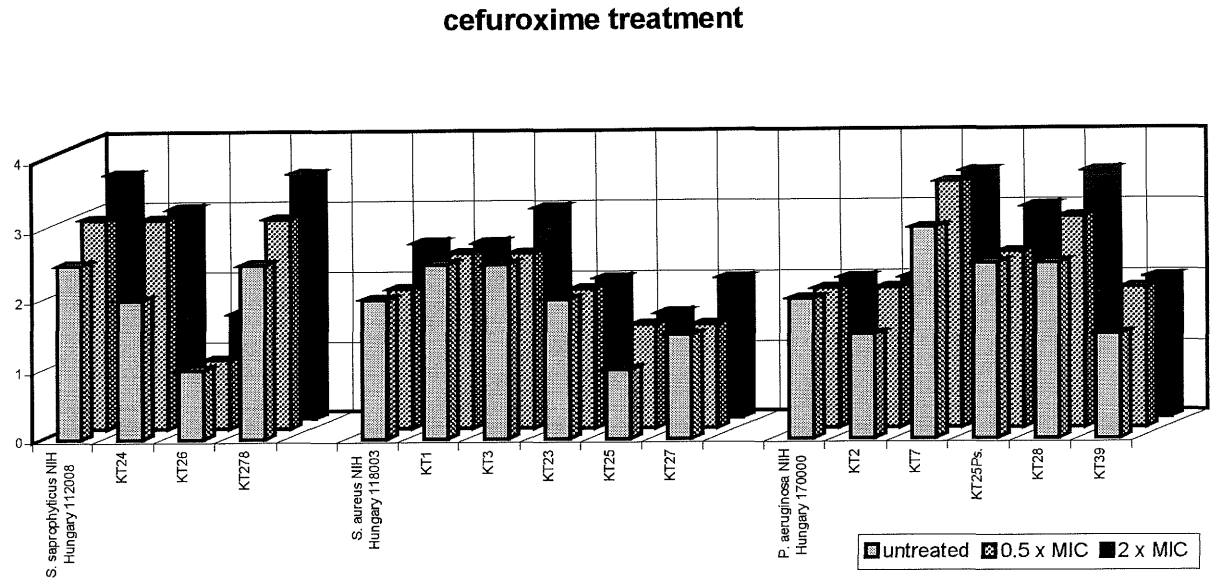


Fig. 3. *(a)* cefuroxime

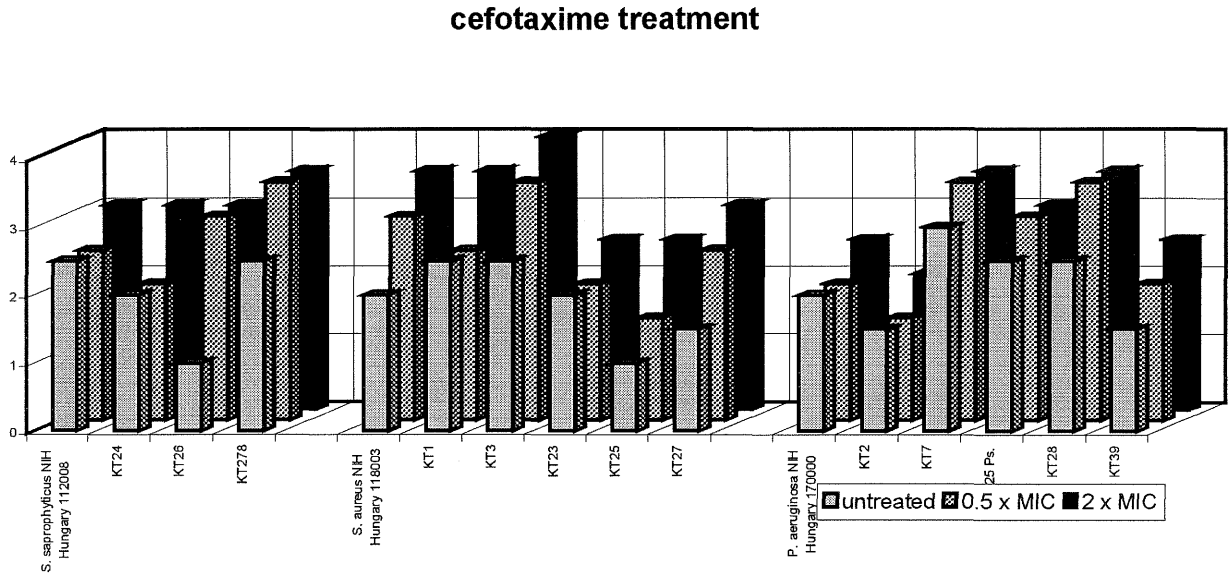


Fig. 3. *(b)* cefotaxime

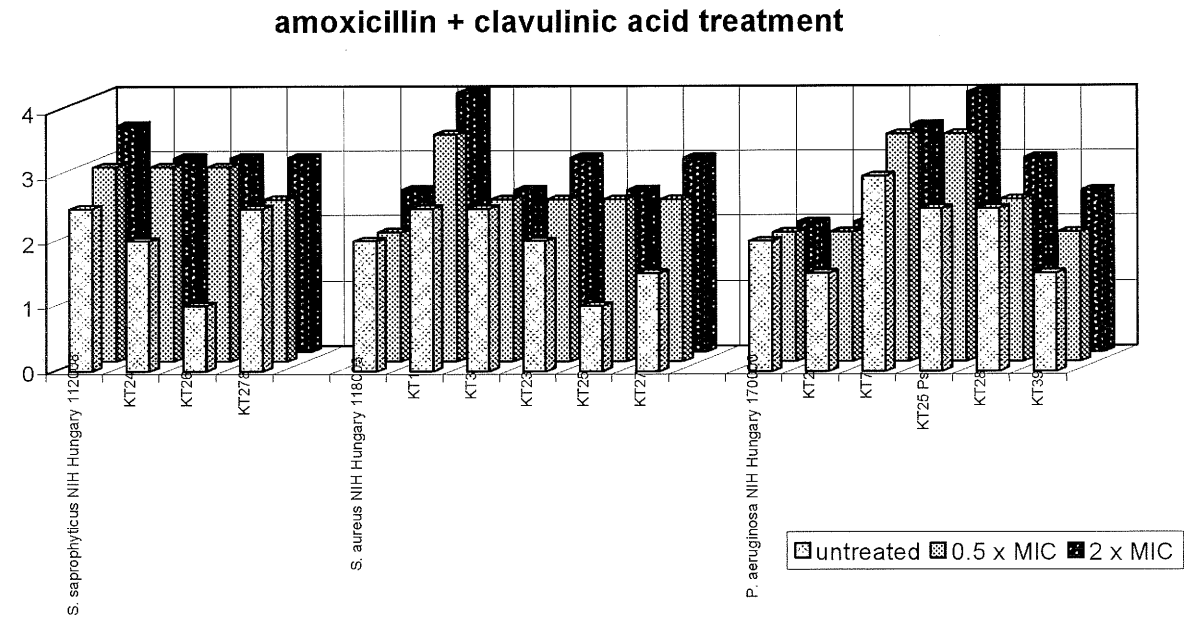


Fig. 3. *(c)* amoxicillin + clavulinic acid

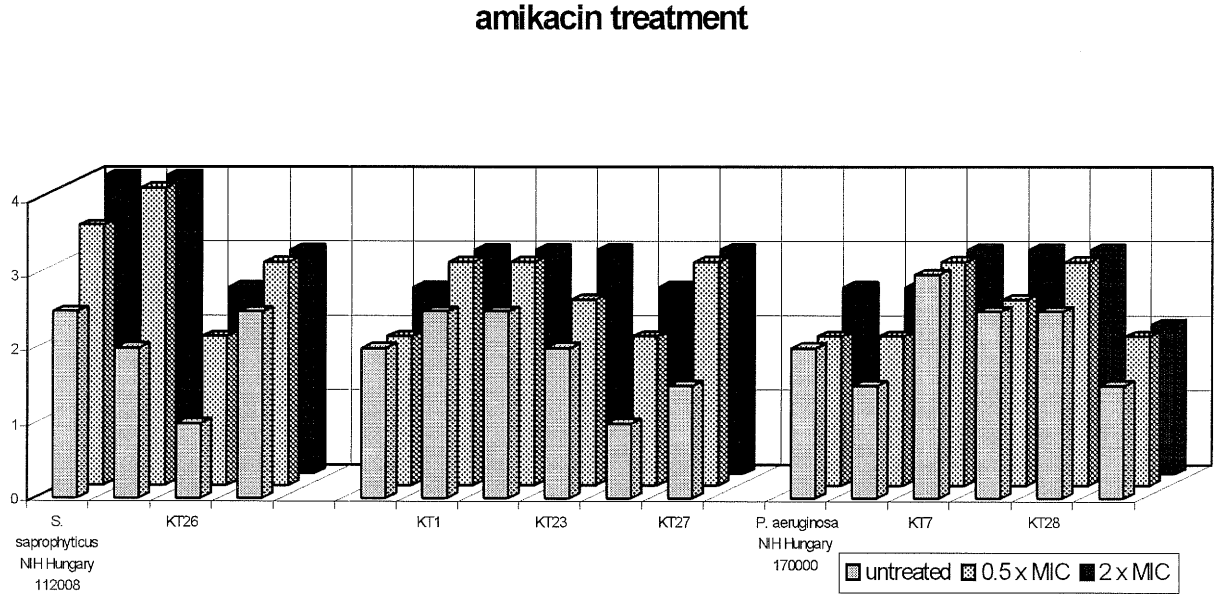


Fig. 3. *(d)* amikacin

Statistical evaluation of the results was performed by one sample one sided Z-test to determine the significant changes of the cell-surface hydrophobicity under antibiotic effect. In Table IX. those *changes* are demonstrated, which reach statistical significance at different control values and at different significance level (size of type I error). In medical research the generally accepted significance level is 1 %, these data were emphasised by bold numbers in the table.

Table IX. Changes in ammonium sulphate concentrations, which reach statistical significance at different control values and significance levels. 1% significance level is marked with bold numbers, as this level is used in the medical research.

Control value (M/L)	***Significance level (size of type I error)***						
	0,10%	*0,50%*	***1%***	*2%*	*2,5%*	*5%*	*10%*
1	0,56	0,47	**0,42**	0,37	0,36	0,30	0,23
1,5	0,85	0,71	**0,64**	0,56	0,54	0,45	0,35
2	1,13	0,94	**0,85**	0,75	0,72	0,60	0,47
2,5	1,41	1,18	**1,06**	0,94	0,89	0,75	0,58
3	1,69	1,41	**1,27**	1,12	1,07	0,90	0,70
3,5	1,97	1,65	**1,49**	1,31	1,25	1,05	0,82
4	2,26	1,88	**1,70**	1,50	1,43	1,20	0,94

We attach here Table X. to show the quantitative results of the salt aggregation test in control (untreated) cases and after antibiotic treatment. The data shown in Table X. are correspond data in Figure 3. According to the statistical analysis more than 39 % of the changes were significant, which are emphasised by bold numbers in Table X.

We would like to emphasise, that the changes meant in all cases the decrease of cell-surface hydrophobicity. Although not all of the changes were significant, but the tendency was the same in all cases: agglutination occurred at higher $(NH_4)_2SO_4$ concentration levels under antibiotic effect.

Table X. Concentration of $(NH_4)_2S0_4$ solutions characteristic to cell surface hydrophobicity of bacteria

	Control	**cefuroxime**		**cefotaxime**		**amoxicillin + clavulinic acid**		**amikacin**	
Bacterial strain		**0.5 x MIC**	**2 x MIC**	**0.5 x MIC**	**2 x MIC**	**0.5 x MIC**	**2 x MIC**	**0.5 x MIC**	**2 x MIC**
S. saprophyticus NIH Hungary	2.5	3	3.5	2.5	3	3	3.5	3.5	**4**
KT 24	2	**3**	**3**	2	**3**	**3**	**3**	**4**	**4**
KT 26	1	1	**1.5**	**3**	**3**	**3**	**3**	**2**	**2.5**
KT 278	2.5	3	3.5	3.5	3.5	2.5	3	3	3
S. aureus NIH Hungary	2	2	2.5	**3**	**3.5**	2	2.5	2	2.5
KT 1	2.5	2.5	2.5	2.5	3.5	3.5	**4**	3	3
KT 3	2.5	2.5	3	3.5	**4**	2.5	2.5	3	3
KT 23	2	2	2	2	2.5	2.5	**3**	2.5	**3**
KT 25 Sta.	1	**1.5**	**1.5**	**1.5**	**2.5**	**2.5**	**2.5**	**2**	**2.5**
KT 27	1.5	1.5	2	**2.5**	**3**	**2.5**	**3**	**3**	**3**
P. aeruginosa NIH Hungary	2	2	2	2	2.5	2	2	2	2.5
KT 2	1.5	2	2	1.5	2	2	2	2	**2.5**
KT 7	3	3.5	3.5	3.5	3.5	3.5	3.5	3	3
KT 25 Ps.	2.5	2.5	3	3	3	3.5	**4**	2.5	3
KT 28	2.5	3	3.5	3.5	3.5	2.5	3	3	3
KT 39	1.5	2	2	2	**2.5**	2	**2.5**	2	2

4.5 Determination of hydrophobicity by the MATH method

Cell surface hydrophobicity of the three standard strains and six clinical isolates (KT 1, KT 25 Sta., KT 24, KT 278, KT 2 and KT 25 Ps.) was determined by the MATH method under different conditions. Quantitative results are summarised in Table XI. Data shown in the table correspond to the percent of hydrophobicity calculated from the spectrophotometrical absorbance of the samples on the basis of the equation given in Section 3.5. *S. aureus* strains showed the highest values of hydrophobicity, coagulase-negative *Staphylococci* (CNS) showed considerably lower levels, while *P. aeruginosa* strains showed the lowest values by this method, respectively. Comparative analysis was performed with three different solvents (hexadecane, toluene, and xylene). Their modifying effect on cell-surface hydrophobicity of standard strains and clinical isolates is shown in figure 4. Effect of cefuroxime, cefotaxime, amoxicillin + clavulinic acid and amikacin at sub-inhibitory (0.5 x MIC) and suprainhibitory (2 x MIC) concentrations was also examined, and results are demonstrated in Figure 5 a and b. Hydrophobic properties of KT 278 strain differed markedly from the other CNS strains, showed similarity with the *S. aureus* strains. This difference can be seen in all figures.

Data were evaluated by **statistical analysis**. Changes were considered significant, if the p-values were less than 0.001. According to the results of ANOVA tests cell-surface hydrophobicity was influenced significantly by the bacterial species ($F=127,254$; $p<0,001$), and by the strain within the bacterial species ($F=63,568$; $p<0,001$). The latter mentioned influencing factor showed only significant effect, because the coagulase-negative KT 278 strain showed altered hydrophobicity properties, than the other CNS strains (similar to *S. aureus* strains).

On the basis of statistical analysis the cell-surface hydrophobicity was not affected significantly by the type of solvents ($F=1,026$; $p=0,437$), by the type of antibiotics ($F=1,429$; $p=0,324$), and by the concentration of antibiotics ($F=0,038$; $p=0,863$).

Figure 4. Modifying effect of three hydrophobic solvents (hexadecane, toluene, xylene) on the cell-surface hydrophobicity of clinical isolates and standard strains determined by the microbial adhesion to hydrocarbons (MATH) method. In the first group hydrophobicity of *S. aureus* strains (NIH Hungary 118003, KT 1, KT 25 Sta), in the second part coagulase-negative *Staphylococci* (NIH Hungary 120008, KT 24, KT 278), and in the third part hydrophobicity values of *P. aeruginosa* strains (NIH Hungary 170000, KT 2, KT 25 Ps) are presented.

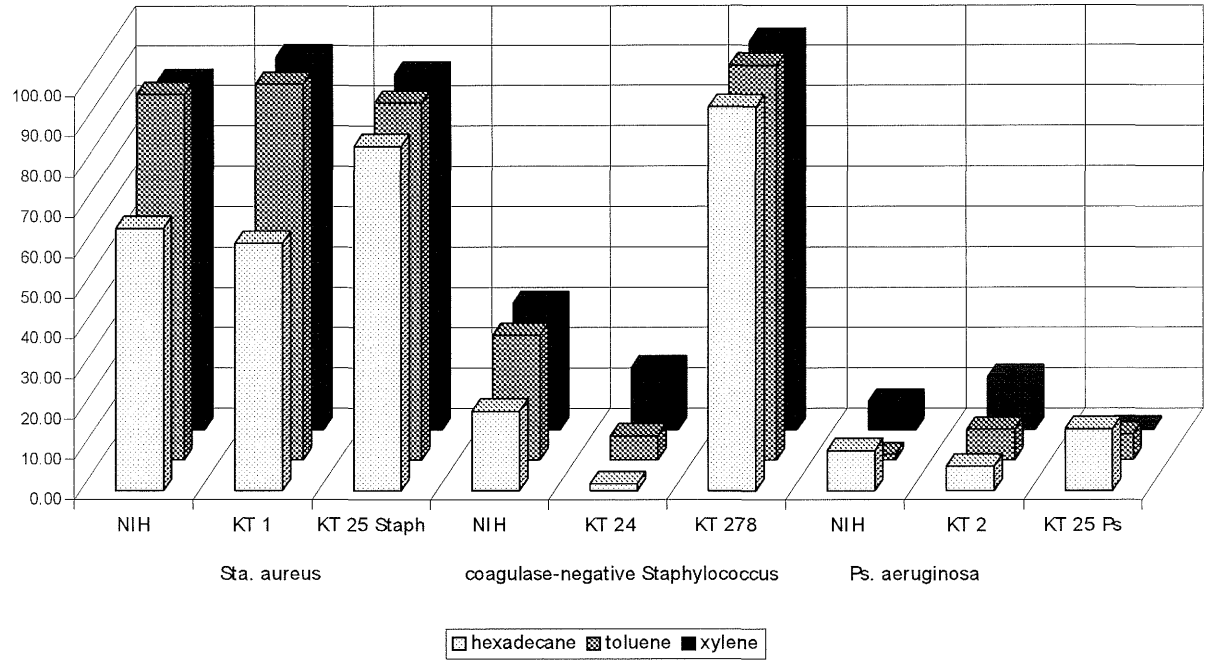


Fig. 4. Clin+St-O

Figure 5. Influencing effect of antibiotics on cell-surface hydrophobicity analyzed by the MATH method. Standard strains and clinical isolates of *S. aureus,* coagulase-negative *Staphylococci* and *P. aeruginosa* groups were treated with the four antibiotics. Percent of hydrophobicity obtained after the administration of sub-inhibitory (0,5 x MIC) and supra-inhibitory (2 x MIC) concentrations of (a) cefuroxime, cefotaxime and (b) amoxicillin + clavulinic acid and amikacin compared to the untreated cases is presented in the figures.

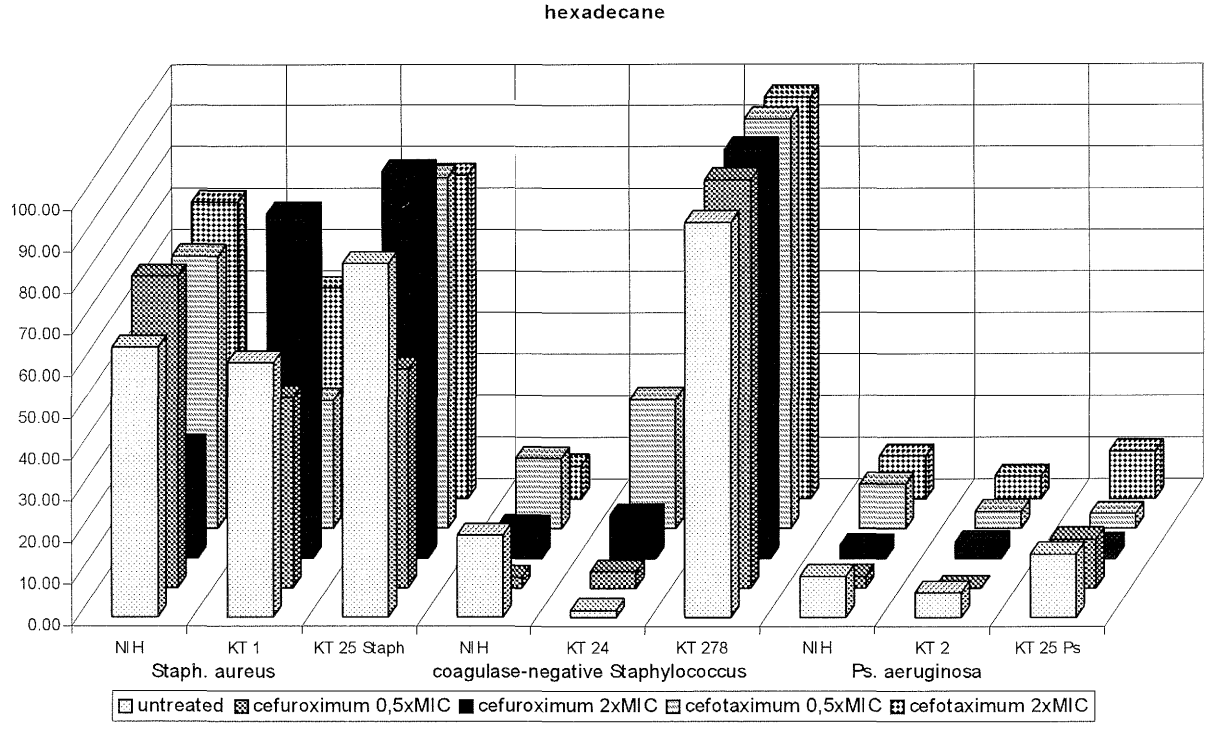


Fig. 5. *(a)* hexadecane + CXM + CTX

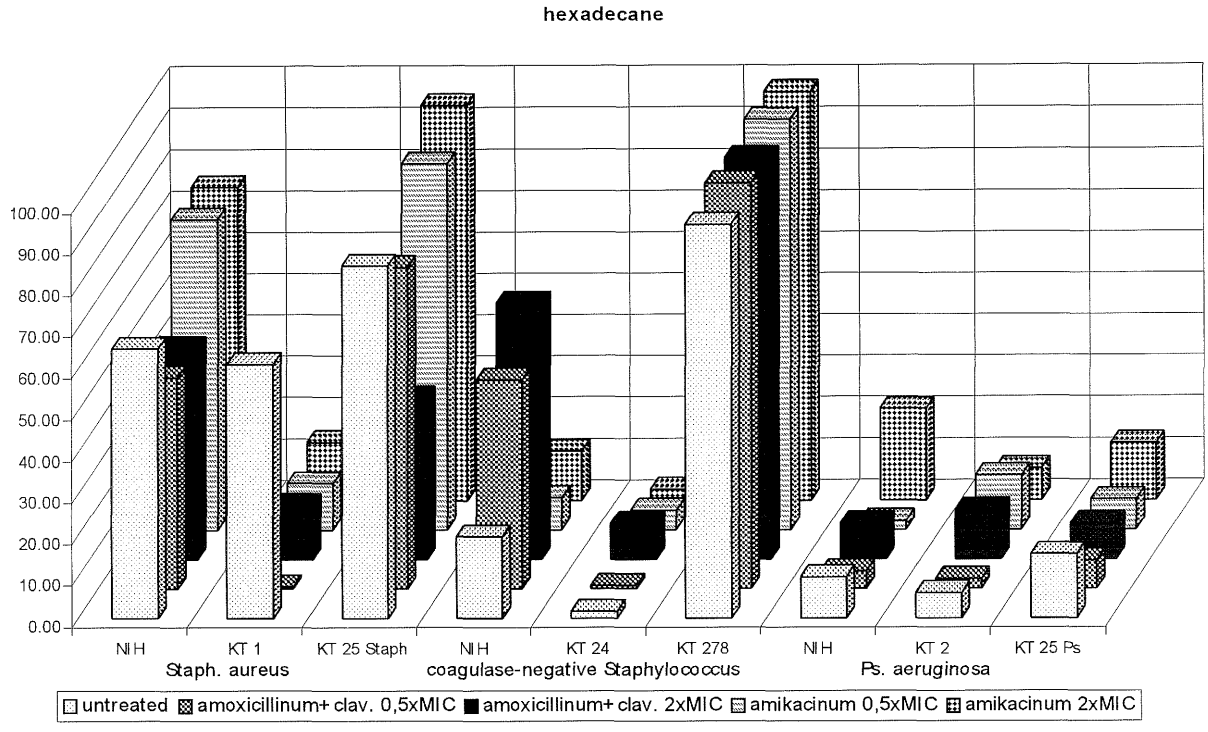


Fig. 5. *(b)* hexadecane + AMC + AMK

Table XI. Cell surface hydrophobicity of the standard strains and clinical isolates determined by the MATH method.

Bacterial strains	Solvent	HYDROPHOBICITY								
		Untreated	cefuroxime		cefotaxime		amoxicillin + clavulinic acid		amikacin	
			0.5 x MIC	2 x MIC	0.5 x MIC	2 x MIC	0.5 x MIC	2 x MIC	0.5 x MIC	2 x MIC
S. aureus NIH Hungary	HEXAD	65.0	75.0	26.9	65.6	71.6	51.0	51.0	75.1	75.9
	TOLU	90.7	91.1	77.8	78.3	90.7	97.9	36.3	85.2	89.9
	XIL	86.4	93.7	52.4	90.3	89.5	78.1	90.4	92.9	91.1
KT 1	HEXAD	61.3	45.8	83.0	30.9	50.8	0.5	13.1	11.3	14.1
	TOLU	93.3	94.3	94.0	93.4	95.3	90.6	89.6	89.4	22.7
	XIL	92.5	88.5	95.2	93.8	95.5	93.1	89.9	90.8	42.9
KT 25 Sta	HEXAD	85.2	52.6	93.2	84.6	78.1	77.7	39.0	88.5	95.3
	TOLU	88.5	96.8	94.6	83.8	89.1	93.6	92.3	91.9	91.9
	XIL	88.2	87.5	95.8	93.6	90.0	97.2	73.4	95.8	97.2
S. saprophyticus NIH Hungary	HEXAD	19.7	2.6	7.5	16.9	0.8	50.4	61.9	7.8	11.9
	TOLU	30.9	17.6	12.8	4.9	1.4	65.5	72.6	27.7	15.4
	XIL	31.4	13.7	10.8	0.3	0.6	59.9	69.2	15.8	10.4
KT 24	HEXAD	1.7	3.9	10.3	31.2	20.7	0.8	8.6	4.7	2.7
	TOLU	6.0	40.0	27.7	29.3	30.3	15	16	7.8	2.4
	XIL	15.2	47.9	40.4	48.6	24.6	14	12	0.2	2.9
KT 278	HEXAD	95.1	98.4	98.5	98.7	97.0	98.0	97.0	99.0	98.6
	TOLU	97.9	99.0	96.3	97.8	97.3	98.0	98.2	94.3	98.0
	XIL	96.4	97.7	98.3	97.4	94.5	97.0	96.8	98.5	95.9
P. aeruginosa NIH Hungary	HEXAD	9.9	2.8	3.0	10.7	10.4	4.2	8.6	2.1	22.1
	TOLU	1.4	3.4	2.7	12.6	27.0	10.3	10.2	0.8	1.6
	XIL	7	5.1	11.8	13.4	25.3	6.2	3.7	4.2	6.1
KT 2	HEXAD	6.0	0.2	3.8	4.1	5.5	2.3	11.8	13.1	7.7
	TOLU	7.6	18.5	22.5	2.6	12.4	13.3	5.2	2.7	16.3
	XIL	13.1	11.4	23.2	2.1	13.0	1.6	6.4	1.2	6.7
KT 25 Ps.	HEXAD	15.3	11.7	3.3	3.6	11.6	6.7	8.6	7.3	13.7
	TOLU	6.4	2.4	10.5	7.7	17.1	1	1	9.6	4.8
	XIL	0.4	2.1	2.5	10	5.1	4	2	9.2	3.7

HEXAD = hexadecane, TOLU = toluene, XIL = xylene

4.6. Capillary electrophoretic analysis of *P. aeruginosa* OMPs:

Capillary electrophoretic OMP patterns of *Pseudomonas* strains were characteristic for the genus and differed markedly (in the number and molecular weights of the dominating proteins) from the profiles of other bacterial genera (*Salmonella minnesota, Shigella sonnei, Escherichia coli, Proteus penneri*). Profiles were reproducible, all measurements were repeated four times. Data indicated in this study are mean values of the four measurements. CE patterns of five *P. aeruginosa* strains isolated from orthopedic wounds were compared to the Hungarian standard *P. aeruginosa* NIH Hungary 170000. On the basis of the molecular weights and ratio of the "major" proteins the comparative analysis of the OMP patterns in the *Pseudomonas* genera was performed. The OMP composition of the six strains examined was similar to each other: all profiles could be characterized by the presence of seven "major" proteins with molecular masses of 22600, 25800, 29100, 34400, 37600, 38800 and 46600, respectively. These proteins were observed in each pattern, but the quantity and relative percentage of the proteins differed in the individual profiles. For example: the quantity of the 38800 rel. mol. mass protein was very high in the KT2 and low in the KT 7 profile. The 29100 protein was presented in low quantity in the KT2 strain, and the 37600 protein in large quantity in the profile of KT 25, comparing to the other *Pseudomonas* strains.

Administration of antibiotics can modify the outer membrane protein composition of bacteria. Treatment of bacteria was made at supra-inhibitory concentrations (2 x MIC) of the antibiotics. In Figure 6 the profiles of KT2 (a), KT7 (b), and KT28 (c) strains are shown in untreated cases and after antibiotic treatment. Results are shown only in the three cases, where significant alterations were observed. Alterations were called significant, if the changes in relative percentage of the protein was higher than the threefold of the standard deviation. (The relative standard deviations of the data are less than 9 %. Since the capillary electrophoretic analysis of real samples *a priori* contains irreproducibility for a certain extent, changes in the protein relative ratios can be considered to be significant if the difference is higher than three times the standard deviation.) In the pattern of KT2 the relative percentage of the protein with relative molecular mass 38800 decreased markedly (from 20% to 6 %) after amikacin

administration. Outer membrane protein composition of KT 7 and KT 28 strains changed also significantly after cefotaxime treatment: the ratio of the protein with rel. mol. mass 38800 increased (from 2 % to 16 % in KT 7, while from 12 to 28 % in the profile of KT 28 strain). Relative peak areas (in percentages) of the seven characteristic proteins in untreated and antibiotic treated bacteria are shown in Figure 7 a and b.

Figure 6. Capillary electrophoresis of outer membrane proteins prepared from untreated (—) and antibiotic treated (----) *Pseudomonas aeruginosa* strains: (a) *KT 2*, (b) *KT 7*, (c) *KT 28*. Major protein components with relative molecular masses of 22600, 25600, 29100, 34400, 37600, 38800, 46600 are marked with 1 to 7, respectively. Details of treatment (*KT 2* bacteria with 25 μg/ml amikacin, *KT 7* bacteria with 100 μg/ml cefotaxime and *KT 28* bacteria with 200 μg/ml cefotaxime) is described in the text. Experimental conditions: buffer, Run Buffer of CE-SDS Protein Kit of BioRad; sample injection, 10-20 psi x sec; voltage, 15 kV; current, 19 μA; detection, 220 nm; temperature, 20°C; capillary, 24 cm length x 50 μm ID, uncoated.

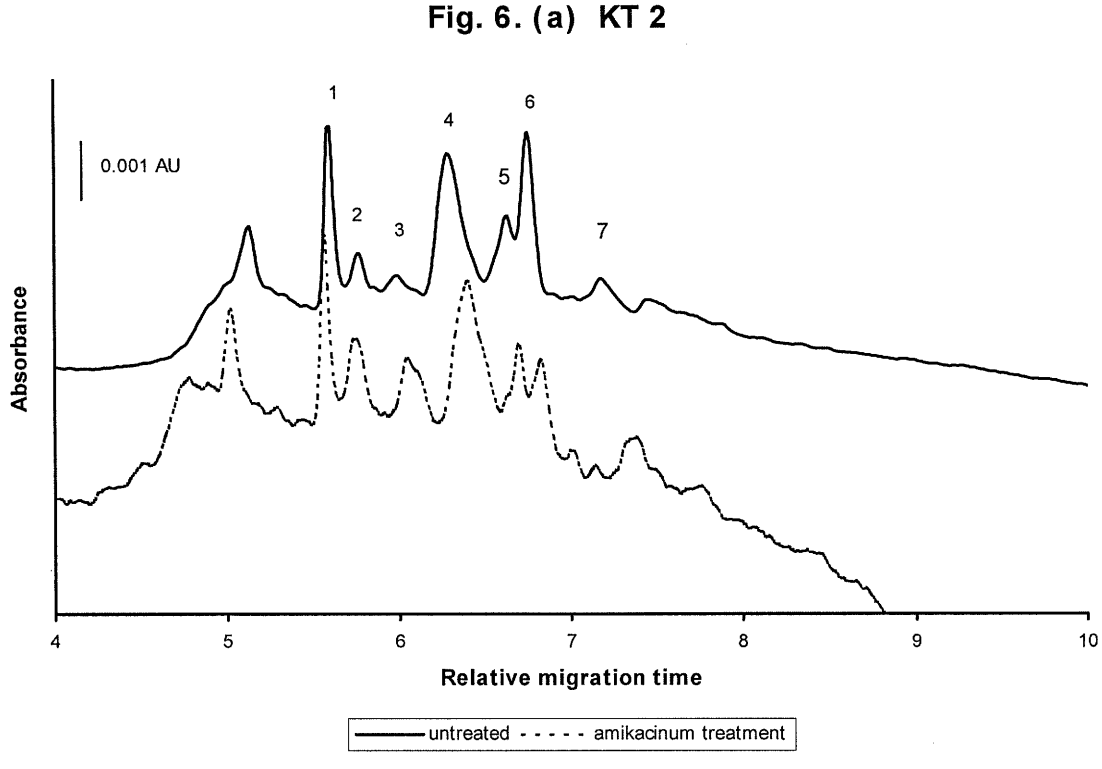


Fig. 6. (b) KT 7

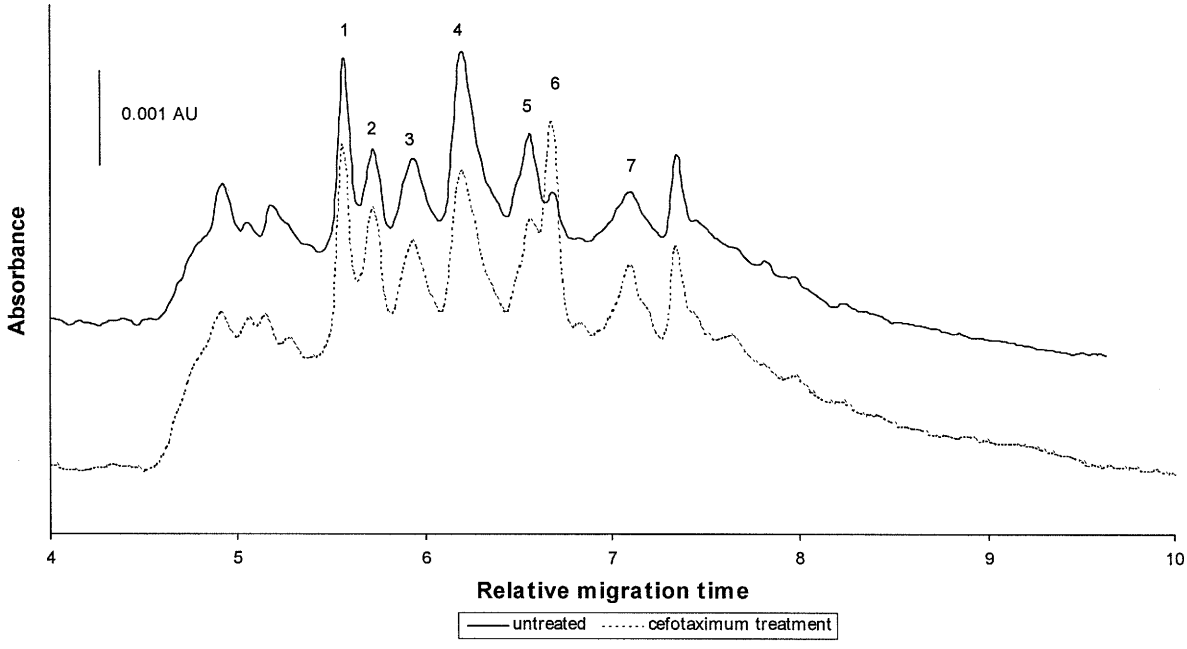


Fig. 6. (c) KT 28

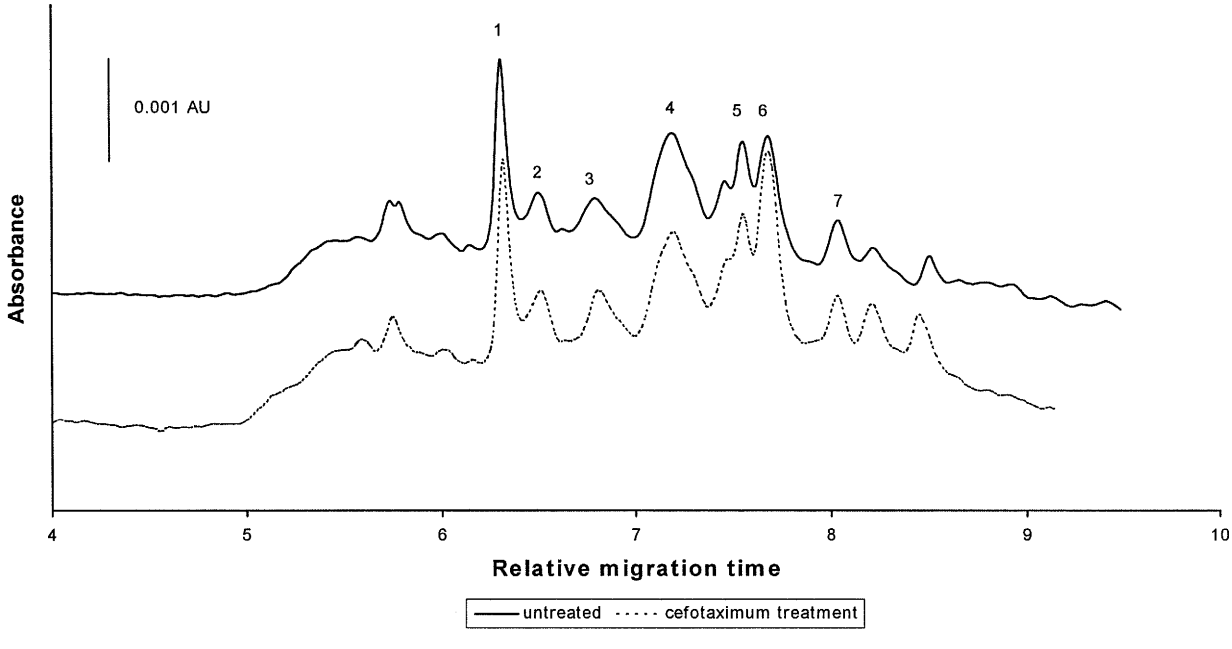


Figure 7. Relative ratio percentages of the seven major components (respective mol. masses 22600, 25600, 29100, 34400, 37600, 38800 and 46600) of the outer membranes of *Pseudomonas aeruginosa* strains (*NIH Hungary 170000; KT 2; KT 7; KT 25; KT 28; KT 39*). Proteins were prepared from untreated and antibiotic treated bacteria. Supra-inhibitory concentrations of Claforan (containing cefotaxime), Aktil (containing amoxicilline and clavulinic acid with a ratio 2:1) and Amikin (containing amikacin) were applied. The relative ratio values were determined from the capillary electrophoretic analyses of outer membrane proteins.

Fig. 7. (a)

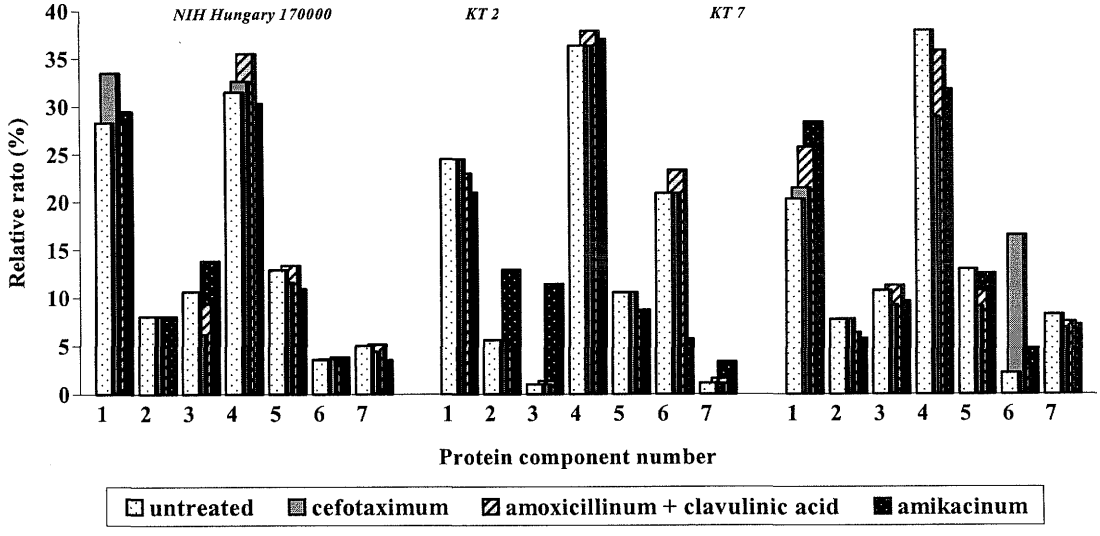
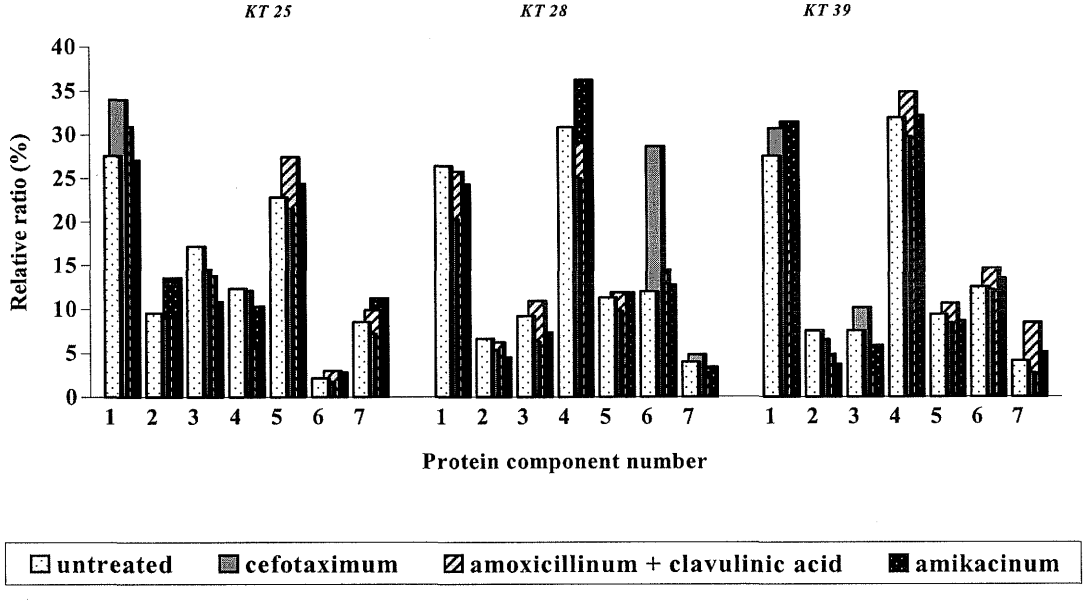


Fig. 7. (b)



4.7 Adhesive ability of bacteria to orthopedic implants:

Adherence of *S. aureus*, coagulase-negative *Staphylococcus* and *P. aeruginosa* strains to the acetabular cup is summarized in Table IX. Beside the three Hungarian standard strains two *S. aureus*, two coagulase-negative *Staphylococcus* and two *Pseudomonas* strains isolated from orthopedic wounds were investigated. The adhesive ability of the *Staphylocccus* strains in untreated cases ($15.67 - 8.80 \times 10^5$ / cm^2) was much higher than the *Pseudomonas* strains ($5.38 - 2.02 \times 10^5$ / cm^2). We could not detect any significant differences between the quantitative adherence of the standard strains and the clinical isolates.

Modifying effect of four antibiotics on the adhesive ability was also analyzed. All four antibiotics were applied in sub-inhibitory and supra-inhibitory levels, respectively. The results presented in Table IX. show that the number of the cells attached to the surface was dependent on the antibiotic treatment. Under antibiotic effect the quantitative adherence of the strains to the medical implant decreased to a certain degree. Considerable alterations are emphasized by bold numbers in Table IX.. It could be observed that the adhesive ability is dependent on the dose of antibiotics – supra-inhibitory (2 x MIC) concentration of antibiotics was able to induce changes in a higher degree.

Data were evaluated by **statistical analysis** (univariate analysis of variance /ANOVA/ and two-way ANOVA /TWANOVA/ tests). Changes were considered significant, if the p-values were less than 0.001. According to the results of ANOVA tests adhesive ability of the strains to orthopaedic implants was influenced significantly by the bacterial species ($F=23,725$; $p<0,001$). Adhesion of *Pseudomonas* strains was much lower, than the *Staphylococcus* strains. The strains within the bacterial species modified the number of attached CFUs also significantly ($F=5,478$; $p<0,001$). Adhesive ability of the *Pseudomonas* strains were similar to each other, but the strains within the *S. aureus* and coagulase-negative *Staphylococcus* group differed from each other significantly.

On the basis of statistical analysis antibiotic treatment was able to modify significantly the adhesion to artificial implants ($F=6,250$; $p<0,001$) in the case of *Staphylococcus* strains. Adhesive properties of *Pseudomonas* strains was characterised by much lower

values in untreated cases as well, and antibiotics could not induce significant changes in these strains. On the other hand we could not detect any significant difference among the four types of antibiotics applied in the study (F=1,724; p=0,173). The concentration of antibiotics influenced significantly the attachment of bacteria to acetabular cups (F= 8,530; p=0,005). Supra-inhibitory concentrations induced much bigger decrease in adhesion than sub-inhibitory concentrations.

Table XII. Number of colony forming units (CFU) of different bacterial strains on the surface of the orthopedic implant. Data presented in the table were calculated to 1 cm^2 of the implant. Each value should be multiply by 10 on the power of 5. The considerable alterations are emphasized by bold numbers.

Bacterial strain	**control**	**cefuroxime**		**cefotaxime**		**amoxicillin + clavulinic acid**		**amikacin**	
		0.5 x MIC	**2 x MIC**	**0.5 x MIC**	**2 x MIC**	**0.5 x MIC**	**2 x MIC**	**0.5 x MIC**	**2 x MIC**
S. aureus NIH Hungary	15.67	13.29	8.63	11.94	8.88	14.62	10.32	**4.63**	**3.77**
KT 1	12.05	8.66	**2.03**	7.83	**1.19**	**3.65**	**1.81**	**6.36**	**4.06**
KT 25 Sta.	8.80	6.13	3.37	6.94	3.76	**2.11**	**1.82**	4.11	**2.50**
S. saprophyticus NIH Hungary	14.34	9.91	**6.14**	13.52	10.44	10.71	8.16	8.67	8.04
KT 24	13.92	**4.35**	**3.13**	8.67	**4.02**	**5.61**	**3.46**	**4.93**	**3.74**
KT 278	12.90	12.81	12.60	10.94	7.71	**5.89**	**3.32**	**6.11**	**5.23**
P. aeruginosa NIH Hungary	2.79	2.63	2.32	2.37	1.79	2.77	2.67	2.52	2.07
KT 2	2.02	1.28	1.17	1.93	1.26	1.77	**1.00**	**1.13**	**0.61**
KT 25 Ps.	5.38	5.11	**2.07**	2.78	**2.00**	**1.13**	**0.83**	4.02	2.50

5. Discussion

Wound infection following elective orthopedic interventions is one of the worst complications in orthopedic surgery. Even in relatively mild cases infection may cause prolonged hospitalization, repeated operations, and often has a strong negative effect on both the patient, and the surgeon as well as on the hospital. This is especially true in cases of total joint replacements, because foreign materials (metallic and polyethylen components, etc.) are built in increasing the risk for wound infection due to the possible biofilm formation on the surfaces [Kocsis, 1996]. Although the incidence of wound infection following total joint arthroplasty gradually decreased to 0.2 to 2 % [Eftekhar and Tzitzikalakis 1986, Espehaug *et al.* 1997, Garvin and Hanssen 1995, Lindwell 1986] its importance is still striking. As to the severity of the complications following wound infection after total joint replacement (removal of the prosthesis, Girdlestone condition, chronic osteomyelitis etc. [Castellanos *et al.* 1998, Laky 1983], chose this fairly hot topic for my study. In order to gain a better understanding of wound infections we used freshly cultured bacteria from orthopedic wound infections and many characteristics were investigated with various microbiological methods.

If wound infection develops early, proper diagnosis is essential. According to data in the literature, if the diagnosis of infection is made following the onset of clinical symptoms in 1 to 2 days, in case of total hip replacement in the first postoperative month, in 50 to 70 % of the cases prosthesis can be saved with debridement and lavage only [Duggan *et al.* 2001, Tsukayama *et al.* 1997]. Making early diagnosis however is not an easy task. The first and most important step is the physical examination [Spangehl et al. 1997]. No examination is known thus far with 100 % sensitivity and specificity, and even the up-to-date image techniques might not provide adequate help, so we had to find other diagnostic procedures. Yet, physical examination is not always sufficient and additional data might be necessary [Abudu et al. 2002], and various laboratory parameters might be useful. Earlier leucocytosis and fever were accepted as of great importance. Nonetheless, it is well known that fever and leucocytosis might be initiated by factors independent of bacterial infections [Than and Málovics, 2000]. Furthermore it was also proved that pathological increase in WBC rarely developed even in the cases of infected total joint

replacements. Canner *et al.* [1984] found higher WBC in only as low as 15 % of their infected cases (8 out of 52).

In the first part of our work a clinical study was conducted. To establish the normal postoperative ranges of laboratory parameters WBC, ESR and CRP were measured in patients who underwent total joint replacement (THR, TKR) and had no clinical symptoms of infection at all. This data could be very helpful for the surgeons in making early diagnosis.

According to the results of our work in patients without complications WBC stays in the normal range in the first 10 postoperative days, which is in correlation with the international literature.

To complete our observations other laboratory parameters were also measured, such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). [Sanzén and Carlson 1989]. CRP is an acute phase protein produced by the hepatocytes, and its concentration in the plasma is increased following trauma and infection. CRP is a sensitive indicator of inflammation, infection or ischemic conditions. Its plasma concentration is increased in the first 48 hours following total joint replacements even in normal non-infected cases and the level returns to normal in 2 to 3 weeks [Niskanen *et al.* 1996]. On the contrary ESR might be elevated for months following surgery. Our results in this study was in complete agreement with the above mentioned data. Increased ESR was continuously present along the first 10 postoperative days, but CRP started to decline from the third postoperative day. So, it was concluded, that among the investigated parameters CRP is the most useful on the early diagnosis in postoperative wound infection. This is clearly shown in one of our patients, who developed postoperative infection. CRP value on the tenth postoperative day was 180 (average is 31.4), while WBC stayed in the normal range (6.76), and ESR was just above the normal value, in non-infected cases (76 mm/h vs. 53.8 mm/h).

We concluded, that sustained increase in CRP even after the tenth postoperative day can be supportive to the diagnosis of wound infection after total joint replacement, if the clinical signs and symptoms suggest infection.

Bacterial infection is a multiphase process including adhesion, colonisation, multiplication, production of bacterial toxins and other virulence factors, invasion and finally the impairment of host tissues. Adhesion of bacterial cells is the initiating step of the process, which is the obligatory condition in the development of an infection. Process of adhesion depends on the cell surface characteristics of bacteria and host tissues. In the first phase non-specific interactions, first of all hydrophobic and electrostatic forces, hydrogen bonds develop, resulting in a **reversible, weak attachment** of bacterial cells.

Among these non-specific forces in this study the hydrophobic interactions, the **hydrophobic properties of bacterial cells** has been analysed. Several components expressed on the surface of bacteria contribute to the hydrophobic character of the cells. (See Introduction for details.) Certain external factors, which are able to modify the expressed biomolecules and thus the cell-surface hydrophobicity may change the hydrophobic interactions and adhesive ability. It is of great significance, that these changes might result in decreased pathogenicity of bacterial strains. In our study the influencing effect of antibiotic treatment on cell-surface hydrophobicity has been investigated.

Several methods have been developed in microbiological practice for determination of hydrophobic properties. In our experiments first the salt aggregation test (SAT) has been applied. The cell-surface hydrophobicity of 13 bacterial strains isolated from orthopaedic samples was examined. In untreated cases aggregation occurred between 1.0 and 3.0 molar concentration (M/L) of ammonium-sulphate. Significant differences between the hydrophobicity of clinical isolates and the Hungarian standard strains could not be observed. Clinical isolates, as well as standard bacteria were treated with sub-inhibitory (0,5 x MIC) and supra-inhibitory (2 x MIC) concentrations of antibiotics (cefuroxime, cefotaxime, amoxicillin + clavulinic acid and amikacin). In several cases changes in the hydrophobicity could be detected under antibiotic effect. The 39 % of these alterations were significant (based on the statistical analysis). Although not all the changes were significant, it has to be mentioned, that the changes in all cases were towards the decrease of cell-surface hydrophobicity (i.e. bacteria became more hydrophilic). Similar results were published by Hostacka and Karelova [1997a] when *Shigella dysenteriae* type 1 strain was treated with aminoglycosides. Braga and Reggio

[1995] found that brodimoprim decreased the surface hydrophobicity of *S. aureus* strains. Significance of these results are that bacterial strains having more hydrophilic surface show decreased adhesion to living tissues (and to medical implants), thus pathogenicity of these strains might be decreased. Another important result, which has to be emphasised, is that supra-inhibitory concentration (2 x MIC) of antibiotics produced more considerable and more frequent changes in bacterial hydrophobicity than sub-inhibitory levels. This phenomenon can draw our attention on the importance of proper antibiotic dosages in clinical cases.

In the next steps of our experimental series another test suitable for cell-surface hydrophobicity analysis has been used: the microbial adhesion to hydrocarbons (MATH) method. Cell-surface hydrophobicity of six clinical isolates (two *S. aureus*, two coagulase-negative *Staphylococci* and two *P. aeruginosa*) causing orthopaedic wound infections and three Hungarian standard strains were determined by the MATH method. Percent of hydrophobicity showed the highest values in *the S. aureus* strains, significantly decreased values in coagulase-negative *Staphylococci*, and lowest values were presented in the case of *P. aeruginosa* strains. According to statistical analysis differences in the hydrophobicity values of these three bacterial species were significant ($F=127,254$; $p<0,001$). Within the *S. aureus* and *P. aeruginosa* groups we could not demonstrate any significant difference between the hydrophobicity of standard strains and clinical isolates, but the hydrophobic characters of the coagulase-negative *KT 278* strain and the *S. saprophyticus NIH Hungary 120008* showed considerable difference. This phenomenon might be explained by the fact, that the strain 278 was *Staphylococcus hominis* identified by biochemical tests.

The hydrophobic solvent applied in the original MATH method by Rosenberg *et al* [1983] was hexadecane. Application of xylene as a hydrophobic solvent was introduced by Sweet *et al* in 1987, and the third hydrophobic solvent added was toluene. According to the statistical evaluation no significant alteration could be detected by using different solvents. This means, that any of the three solvents might be used during the hydrophobicity studies (under same experimental conditions).

Effect of different types of antibiotics (cefuroxime, cefotaxime, amoxicillin + clavulinic acid and amikacin) on cell-surface hydrophobicity was also analysed in our study by

MATH technique. Although several changes could be observed after antibiotic administration, on the basis of statistical analysis non of the antibiotics were able to induce significant alterations (F=1,429; p=0,324). Influencing effect of antibiotic concentration was also examined at sub-inhibitory (0,5 x MIC) and supra-inhibitory (2 x MIC) levels. The differences in hydrophobicity measured after administration of 0,5 x MIC and 2 x MIC concentrations of the four antibiotics were not significant (F=0,038; p= 0,863). It is known, that antibiotic treatment may modify bacterial morphology and several functions of cells. It can be presumed, that those cell-surface structures, which are involved in the MATH method were not considerably modified by these types of antibiotics, therefore we could not demonstrate significant changes of hydrophobicity with this technique.

Comparing data obtained by the MATH and SAT techniques, we could not detect any correlation between the results of the two tests. Similar consequences were published by Ocana et al, 1999. It might be explained by the fact, that probably different structures and properties of the bacterial surface are involved in each technique employed [Ocana *et al,* 1999].

The reversible attachment of bacterial cells is followed by the **permanent adhesion** of microorganisms. This phase of attachment develops due to the specific interactions between the bacterial surface components and the receptors of host tissues (or artificial biomaterials). Several bacterial components expressed on the surface might be involved in this phase of infection, e.g. fimbrial proteins, non-fimbrial adhesins, lipopolysaccharides, OMPs, etc. Among these factors the outer membrane proteins have been analysed in details in our study. These proteins are presented in the cell wall of Gram-negative bacteria, playing important role in bacterial cell functions (See Introduction for details). OMP composition of the standard *P. aeruginosa*, and the five *Pseudomonas* strains from orthopaedic sources were examined by capillary electrophoresis. This method was already applied for bacterial protein analysis in the Dept. Medical Microbiology and Immunology, University of Pécs. Capillary electrophoretic patterns of the outer membrane proteins of *Pseudomonas* strains were characteristic for the genus and differed markedly from the profiles of other bacterial

genera. Several data can be found in the literature about *P. aeruginosa* outer membrane proteins. Hostacka and Karelova [1997d] described four significant outer membrane proteins with relative molecular masses of 23000, 35000, 38000 and 45000 by SDS-PAGE. Hancock and Nikaido [1978] found four major protein bands with rel. mol. masses of 17000, 21000, 35000 and 37000 in the outer membrane fraction of *P. aeruginosa PAO1* (separated in the absence of EDTA). In our capillary electrophoretic patterns of *Pseudomonas* strains seven major proteins were observed. The relative amounts of proteins, however, were not identical in each profile, and some major proteins in certain patterns appeared in low amounts.

Administration of antibiotics can modify the outer membrane protein composition of bacteria (see our previous results in I. Kustos *et al.* [1998b]). The three antibiotics (cefotaxime, amoxicillin/clavulinic acid (2:1), and amikacin) applied in the experiments affected the outer membrane composition unevenly. Significant changes, (*i.e.,* differences in the relative amounts of proteins higher than three times the standard deviation) were found in three cases, but smaller variations were also observed. The bacterial strains responded diversely to the different antibiotics. Since the antibiotics may influence the protein synthesis or enzymes' functions the observed changes in the outer membrane composition upon antibiotic treatment are not surprising. Noteworthy changes were induced by cefotaxime in the membrane compositions of *KT 7* and *KT 28*. Similarly, amikacin altered the outer membrane profiles mostly in the *KT 2* strain, where a strong decrease in hydrophobicity was observed upon the treatment. The disproportion between the relative amounts of the proteins induced by amikacin, (a protein-synthesis inhibitor) must be further investigated. The demonstrated significant changes might be considerable, because alterations of the OMP composition may influence the interactions between bacterial components and host receptors, and so the process of adhesion (in this case the second or irreversible phase) might be altered after antibiotic treatment.

It has to be emphasised, that changes in the cell-surface hydrophobicity were more often detected after antibiotic treatment than changes in capillary electrophoretic OMP profiles. This might be explained by the fact, that hydrophobic characters are regulated by more factors: e.g.: fimbrial proteins, lipopolysaccharides, lipids, phospholipids, M protein in *Streptococcus pyogenes*, 259 kDa surface protein in *Streptococcus gordonii*, spore

coats in *Bacillus* and *Clostridium* species, etc. [Doyle, 2000]. In all cases when OMP profiles were modified by antibiotics, alterations in cell-surface hydrophobicity could also be detected.

In different fields of modern health care system the increasing application of **implantable biomedical devices**, such as prostheses, several types of catheters and tubes resulted in a significantly enhanced number of implant associated infections. Increasing incidence of postoperative infections might be (partly) related to the implantation of artificial devices, since bacteria show higher adhesive tendency to non-living substances, than to living tissues. Adhesion of microorganisms to the surface of artificial objects or to living cells occurs similarly. Adhesion is followed by colonisation, and **biofilm formation** on the surface of medical implants. During biofilm formation an exopolisaccharide glycocalyx matrix is produced by the bacterial cells. Multiplication of bacterial cells occurs in this matrix on the surface of implants. Life processes within the biofilm matrix have been intensively studied recently. Within the biofilm special, hardly reproducible circumstances are present. Diffusion of the oxygen, transport of the nutrients, and removal of the metabolic end-products may vary in time and between different parts of the biofilm. Metabolic processes are slowed down, and the growth rate is really slow [Anwar *et al.*, 1990, Costerton *et al.,* 1995]. Another important phenomenon, which has to be mentioned is the communication of the bacterial cells within the glycocalyx matrix, resulting in the rearrangement of the biofilm structure. Further clinical problems might originate from the detachment of bacterial cells from the surface of biofilm, getting again to planctonic phase, reaching new surfaces in the host, and causing severe clinical symptoms, as sepsis. The exopolisaccharide matrix ensures protection for the microorganisms against the antibiotics, enzymes, biocides [Kétyi 1989] and the immune system of the host, like phagocytic cells or antibodies. According to Kétyi [1990] biofilms in mice showed 25-32 times higher resistance level against antibiotics (streptomycin, chloramphenicol) than the planktonic phase of the same bacterial strains. Among biocides the same phenomenon could be observed in cases of sodium hypochlorite and silver nitrate, with an enhancement of 10-16 times. In summary, presence of biofilm might result in very resistant infections, with the consequences of reoperations, removal of the devices, and further financial costs. Several microorganisms

have already been detected in biofilms. Practically all types of bacteria may participate in biofilm formation, but there are some bacterial strains, which prone to form biofilms more frequently, such as *Staphylococcus epidermidis, Streptococcus mutans, Pseudomonas aeruginosa* or *Bacteroides fragilis.* Treatment of a fully developed bacterial biofilm is rather difficult. Recently intensive research efforts have been made on the selection of proper chemical composition and on proper shape of biomedical devices. Pretreatment of the surface of implants (e.g. with surfactants), or mixing antibiotics (e.g. fluoroquinolons) or disinfectants in the material of catheters have been performed as preventive steps.

In orthopaedic surgery the increasing application of joint prostheses might also been accompanied by bacterial infections. Bacteria are usually situated in biofilms on the surface of prostheses, thus the phenomenon of biofilms has a great significance for the clinicians as well, and importance of biofilm formation has to be studied accurately. In the present study adhesion of bacterial strains causing orthopaedic wound infections to acetabular cups has been examined. First step of the infectious process – adhesion – was investigated (this was performed by application of short incubation time). Adhesive properties of the different bacterial species showed significant differences ($F=23,725$; $p<0,001$). The highest number of CFUs on the surface of the implant could be observed in the case of CNS strains, the *S. aureus* strains showed lower adhesive ability, while the *Pseudomonas* strains could be characterised by the lowest values. Modifying effect of four antibiotics (cefuroxime, cefotaxime, amoxicillin + clavulinic acid and amikacin) on the adhesion to acetabular cup has been also analysed. Antibiotic treatment was able to induce significant changes in the number of attached bacterial cells ($F=6,250$; $p<0,001$), independently from the type of antibiotics. Supra-inhibitory (2 x MIC) concentrations of antibiotics induced a significantly greater decrease in the attached CFUs, than 0,5 x MIC concentration ($F= 8,530$; $p=0,005$). It has to be mentioned that supra-inhibitory antibiotic concentrations may also have bactericidic effect, but the c.f.u. was checked at the end of one hour long antibiotic treatment and was diminished only slightly in comparison with untreated control. The bactericid effect might contribute to the decrease of colony forming units after administration of antibiotics in 2 x MIC concentrations. The other

reason of decreased number of c.f.u. was the diminished adhesive capability of antibiotic modified bacterial cells.

Correlation between cell-surface hydrophobicity and adhesion to artificial surfaces was demonstrated in our study as well. Changes in the cell-surface hydrophobicity of bacterial pathogens under antibiotic effect (determined by the salt aggregation test) were accompanied in all cases by decreased adhesive ability to the orthopaedic implant. Supra-inhibitory concentrations of antibiotics could cause more often a stronger alteration in hydrophobicity and also in adhesive ability. Considerable decrease could be also observed in the number of attached microorganisms, when the OMP composition of *Pseudomonas* strains showed significant alterations after antibiotic treatment. Remarkably, adhesive ability of bacterial strains changed in more cases than the cell-surface hydrophobicity. This phenomenon might be explained by the fact, that bacterial attachment and colonisation are dependent on several factors: hydrophobic interactions electrostatic attraction, hydrogen bonds, specific interactions between bacterial antigens and surface receptors, production of the extracellular polysaccharide etc. [Deighton *et al*, 1990].

Summarising the consequences of our study, bacterial components must interact with the surface receptors of host tissues or medical implants to perform attachment and colonisation [Christensen *et al*, 1985]. Any changes in the surface composition of the bacterial pathogen may cause dramatic alterations in its adhesive ability [Anwar *et al*, 1990] and furthermore in its pathogenicity. Antibiotic treatment was able to induce significant changes in the cell-surface hydrophobicity of orthopaedic bacterial isolates, which might influence the first step of adhesion. Outer membrane protein composition was also altered under antibiotic effect, this might have a modifying effect on the second, permanent phase of attachment. We could also demonstrate changes in the adhesion to acetabular cups under antibiotic effect. Based upon these data we conclude, that proper perioperative prophylaxis and antibiotic treatment might be able to diminish the possibility of postoperative wound infections in orthopaedic patients. It also has to be emphasised, that supra-inhibitory concentration of antibiotics was much more effective in altering bacterial surface properties and function, therefore proper dosage of antibiotics has to be determined and applied during the treatment of orthopaedic patients.

6. Conclusions

1. Among the examined laboratory parameters (ESR, WBC, CRP), CRP seems to be the most useful one for the orthopedic surgeon in case of an early wound infection after total joint replacement. In "normal" non-infected case its level tends to go back to normal after the 3rd postoperative day. Further increase in the CRP level after the 3rd postoperative day supports the diagnosis of early wound infection.
2. We isolated and identified five *Staphylococcus aureus*, three coagulase-negative *Staphylococcus* (e.g.: *S. hominis*) and five *Pseudomonas aeruginosa* strains from orthopedic wounds.
3. The minimum inhibitory concentration (MIC) values were determined by the tube dilution method. In this investigation four antibiotics (cefuroxime, cefotaxime, amoxicillin combined with clavulinic acid, amikacin) and 16 strains (three standard and 13 orthopedic isolates) were used. The values are given in μg/ml. (See table VIII.)
4. Cell surface hydrophobicity of the isolated strains and the three Hungarian standard bacterial strains were determined by salt aggregation test (SAT) and microbial adhesion to hydrocarbons method (MATH). Refering to the results of MATH tests, we could not detect any difference between the three solvents: toluene, xylene, and hexadecane. Comparing data obtained by the SAT and MATH similarly to the literature, we could not observe any correlation between the results of the two tests. It was found that the results received by SAT method showed better correlation with the data obtained in other experiments (OMP analysis, determination of bacterial adherence).
5. Clinical isolates, as well as standard bacteria were treated with sub-inhibitory (0,5 x MIC) and supra-inhibitory (2x MIC) concentrations of antibiotics (see point 3). It was concluded, that the cell surface hydrophobicity decreased under antibiotic effect. The supra-inhibitory concentration induced more considerable decrease in hydrophobicity than sub-inhibitory level.

6. We extracted and analysed the outer membrane proteins (OMP) of *Pseudomonas aeruginosa* strains by capillary electrophoresis. Antibiotics (see point 3) were able to modify the OMP composition of *Pseudomonas* cells. It has to be emphasized that changes in cell surface hydrophobicity were more often detected after antibiotic treatment than changes in capillary electrophoretic OMP profiles.
7. The adhesive ability of bacterial strains to orthopedic implants was analysed by the sonication method. The CNS strains were most active in adherence to orthopedic prostheses. *S. aureus* strains showed lower, and *Pseudomonas* strains the lowest adhesive ability. Antibiotic treatment could decrease the number of attached bacterial cells, independently from the type of antibiotics. Supra-inhibitory (2 x MIC) concentrations of antibiotics induced significantly greater decrease in the attached CFU.
8. We concluded, that OMP composition, cell surface hydrophobicity and adhesive ability of bacterial strains to prostheses are in correlation with each other. Antibiotic treatment was able to induce changes in the OMP profile, reduce hydrophobicity and decrease the adhesive ability to orthopedic implants. But to tell the truth sometimes other factors might also contribute and influence the bacterial adherence to the surface of prostheses.
9. Aseptic operation and antibiotic prophylaxis (before and shortly after operations) could diminish the wound contamination, and infection of orthopedic implants. In the case of wound infection the proper choice and proper dose of antibiotics can reduce the number of the late infectious complications of orthopedic interventions.

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