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Biotechnological Methods for Precise Diagnosis of Methicillin Resistance in *Staphylococci*

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Abstract: *Antimicrobial resistance is one of the most urgent problems in medicine nowadays. The purpose of the study was to investigate the microorganisms resistant to first-line antimicrobials, including gram-positive cocci, particularly the methicillin-resistant Staphylococcus aureus and coagulase-negative Staphylococci, the major agents of nosocomial infections. Owing to the multi-resistance of these agents, precise diagnosis of the methicillin resistance of Staphylococci is of greatest clinical importance. It is not enough to use only conventional microbiological diagnostic methods. Biotechnological methods should be also involved. In our studies, the following methicillin resistance identification methods were used: the disk diffusion method, detection of the mecA gene by PCR, E-test and Slidex MRSA test. For molecular typing, PFGL, RAPD tests and detection of the coa gene were used. All the MRS strains were multiresistant to antibacterials. No vancomycine resistance was registered.*

Keywords: *Microorganisms, Resistance, Methicillin resistance, Antimicrobial agents, Staphylococci, Biotechnological methods*

Introduction

The current era of antimicrobial therapy began more than 60 years ago. The use of antimicrobial agents – antibiotics and chemical drugs has greatly contributed to improvements in health. However, the use of antimicrobial agents has posed a serious problem – the increasing prevalence of microorganisms that have acquired the so-called "antimicrobial resistance". The continuing emergence of pathogenic microorganisms, which are resistant to first-line antimicrobials, is the course of an increasing concern.

The clinical consequences of such a resistance embrace documented increase in morbidity, therapeutic failure or relapse and mortality. So, they lead to the impairment of the quality of life in patients.

Antimicrobial resistance not only affects patients, but also increases the burden on health care services as it has certain economical consequences and causes additional health and medical care costs because of a prolonged hospital stay, the use of alternative drugs, which may be more expensive and/or even potentially more toxic, additional diagnostic testing, etc.

The strategies for prevention and control of antimicrobial resistance are complex and include the establishment of accurate surveillance systems based on precise diagnostics, generating valid, reliable and comparable data on the incidence, prevalence and modes of spread of resistant microorganisms, prudent use of antimicrobials, implementing of hygiene and infection control standards in health care facilities, co-ordination among the human, veterinary and environmental sectors, etc. [4, 6, 13, 15].

Since antimicrobial resistance spreads mainly among hospital microbial flora, i.e. the agents of nosocomial infections, first of all, infection control and surveillance systems should be established there.

Among the pathogens causing hospital infections, gram-positive agents, particularly *cocci*, have become predominant over the past two decades. From the numerous genera of gram-positive bacteria, three of them, i.e. *Staphylococci*, *Streptococci* and *Enterococci*, have become increasingly important causes of nosocomial infections in patients [2, 3, 9].

Over the past few years, *Staphylococci* have emerged as the most prevalent cause of hospital-acquired infections both in immunological healthy and immunosuppressed patients. This is due to the increased incidence of serious *S. aureus* infections with high mortality and complication rates associated with these infections as well as the increased incidence of infections caused by the coagulase-negative *S. epidermidis* group *Staphylococci* (CoNS), particularly catheter-related infections. They have become widespread since the 1980s [17, 21, 23].

The major problem connected with the chemotherapy of staphylococcal infections is the selection of resistant microorganisms.

Penicillin resistance became widespread shortly after penicillin, the first antibiotic, was introduced into clinical use in the early 1940s. As early as in 1944, reports described isolated strains of *S. aureus* that were resistant to penicillin by virtue of the production of the enzyme penicillinase (in the 1960s, it was renamed β-lactamase). The incidence of resistance was initially low, but increased over the years, until, by 1960, penicillin-resistant *Staphylococci* represented a vast majority of strains in many hospitals.

Nowadays, this form of resistance is commonplace. There are reports that as many as 90% of the isolates of *S. aureus* are resistant to β-lactamase labile penicillins. Many coagulase-negative *Staphylococci* are also able to produce β-lactamases and are therefore resistant also to the action of β-lactam antibiotics.

To overcome the failure of β-lactam therapy, semisynthetic penicillinase-stable penicillins such as oxacillin and methicillin were synthesized. Methicillin (oxacillin), introduced in 1961, proved effective against nearly all penicillin-resistant strains. Once again, shortly after the introduction of methicillin into clinical use, methicillin-resistant *S. aureus* (MRSA) was isolated. On this occasion, the resistance was not due to the production of an enzyme that modifies the drug, but because of the presence of an altered penicillin-binding protein PBP2a. The gene responsible for this property has been designated *mec*A [8, 16, 19].

Methicillin resistance has spread also to the coagulase-negative *Staphylococci*. Methicillin-resistant *S. epidermidis* (MRSE) and other representatives of the CoNS group such as *S.*

haemolyticus, *S. warneri*, *S. hominis*, etc. are often clinical isolates [7].

The most typical feature of methicillin-resistant *Staphylococci* (MRS) is their multiresistance. The most strains of MRS are resistant to erythromycin, gentamicin, tetracyclin and other antimicrobial agents [18, 19].

This study was undertaken to present data on the prevalence of MRS in a surgical hospital in Riga, to make more precise the protocol for methicillin susceptibility testing by biotechnological methods and analysis of the antimicrobial susceptibility of methicillin-resistant *S. aureus* and coagulase-negative *Staphylococci* to the panel of antimicrobials.

Materials and methods

The study was carried out in a surgical 250-bed hospital with an intensive care unit during 1998-2003.

Bacterial strains

More than 3400 clinical isolates of *Staphylococcus* spp. were studied.

Most of these isolates were from wounds, skin, abscesses, followed by blood, indwelling artificial devices, throat, etc. All isolates were gram-positive catalase-positive clustering *cocci*.

1704 coagulase-positive isolates identified as *S. aureus* were included, 56 of them methicillin-resistant and 1782 coagulase-negative *Staphylococci*, identified as representatives of *S. epidermidis* or *S. saprophyticus* groups, 130 of them methicillin-resistant. Most often *S. epidermidis sensu stricto* was isolated, followed by *S. haemolyticus*, *S. hominis*, *S. warneri*, *S. capitis*, etc.

Staphylococci were identified by conventional tests such as coagulase, phosphatase, hemolysis, susceptibility to novobiocin, acid production from maltose, mannitol, etc. and the automated BBL Crystal system (Becton – Dickinson) [17].

Antimicrobial susceptibility testing

Antimicrobial susceptibilities for all isolates were tested by the disk diffusion method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) using Mueller-Hinton agar (MHA, Oxoid, UK) against the following panel of antibiotics: penicillin, gentamicin, cefazolin, erythromycin, clindamycin, vancomycin, ciprofloxacin, trimethoprim-sulfamethoxazole.

Methicillin (oxacillin) susceptibility testing

- ***The standard agar diffusion technique*** with commercial disks (BBL Microbiology Systems) was used. An oxacillin disk with a potency of 1 μg of oxacillin was used for detection of methicillin resistance. Because of the difficulties in detecting cross-resistance among the penicillinase-resistant penicillins (methicillin, oxacillin), the oxacillin disk is now the recommended choice for detecting methicillin-resistant *Staphylococci*. Plates were incubated at 35°C for 24 h, when zones of incubation were measured. Plates with *S. epidermidis* cultures were incubated for 48 h.

S. aureus ATCC 29213 was used as a methicillin-susceptible control strain, NCTC 8325 as a

methicillin-resistant control strain.

• ***E-test***
E-test (AB Biodisk) was used as a quantitative technique for determination of antimicrobial susceptibility and minimal inhibitory concentration (MIC). Mueller-Hinton medium, supplemented with 2% NaCl, was used for inoculation. Incubation time with E-test strips was 24 h and 48 h for *S. aureus* and CoNS, respectively [1].

• ***Detection of the methicillin resistance gene mecA by PCR (polymerase chain reaction)***
Staphylococcal chromosomal DNA was extracted by the lysostaphin-CTAB method [12].

PCR was performed with the following primers, previously designed by [10]: *mec*A1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and mecA2 (5' - CCA ATT CCA CAT TGT TTC GGT CTA A). DNA amplification contained 30 cycles ("Progene", Techne, UK). A positive result was indicated by the presence of the 310-bp amplified DNA fragment revealed by electrophoresis on a 1.5% agarose gel.

Results were obtained within 4 h. Each PCR included a methicillin-resistant strain as a positive control and distilled water as a negative control.

• "***Slidex MRSA" detection***
The commercial Slidex MRSA detection kit (BioMerieux, Lyon, France), a latex agglutination test detecting the production of PBP2a, encoded by the *mec*A gene was applied, thus allowing a rapid detection of methicillin resistance in *S. aureus*. It is regarded as a simple, rapid (20 min) and accurate method, which can be used for diagnostic, surveillance, epidemiological and research purposes. The kit is not validated for coagulase-negative *Staphylococci*, although the mechanism of methicillin resistance in CoNS is similar to that in coagulase-positive *S. aureus*.

Colonies identified as *S. aureus*, *S. haemolyticus*, *S. epidermidis*, *S. hominis* and *S. capitis* were taken from blood agar plates after the growth for 18-24 h at 35^{o}C. Further PBP2a extraction and latex agglutination procedures were performed in accordance with the kit instructions. If PBP2a is present in the cell, a specific agglutination reaction with monoclonal antibody-sensitized latex can be observed.

Molecular typing methods
Three methods were used for molecular typing of isolated *Staphylococci* strains.

• ***Macrorestriction analysis by Pulsed-Field Gel Electrophoresis***
Restriction fragment length polymorphism typing is based on the randomness of the distribution of restriction endonuclease cleavage sites on the bacterial genome. Genome restriction, followed by Pulsed-Field Gel Electrophoresis (PFGE), is currently the typing method of choice for MRSA and for most other bacterial pathogens. It has been agreed to be a "golden standard" method owing to its high discriminatory power and reproducibility. However, the method is cumbersome and requires a high practical experience in DNA sample preparation.

In this study, strains of *S. haemolyticus* were analysed using *SmaI* macrorestriction and PFGE. The analysis was performed at the Robert Koch Institute (RKI), Wernigerode by the research

group of W. Witte. In short, bacterial cells were inoculated and lysed in agarose blocks according to a standardized protocol. After incubation with endonuclease *SmaI*, the blocks were loaded in 1% agarose gel. PFGE was run on a BioRad CHEF II apparatus for 26 h.

• ***Randomly Amplified DNA Polymorphism Analysis (RAPD)***
Genomic DNA was isolated from *Staphylococci* colonies by using a DNA extraction kit (MBI "Fermentas", Lithuania). After dilution to the concentration 5 ng/µl, bacterial DNA was added to the PCR mix, containing one of four primers. The target sequences were amplified in a "MJ research" thermal cylinder in 25-µl reaction volumes (after initial denaturation at 94°C for 2 min) for 45 cycles (94°C, 30 s, 32°C, 30 s, 72°C, 1 min 30 s), followed by the final extension step at 72°C for 5 min. Amplified fragments were resolved in 1.5% agarose gels and analysed using the "GelCompar4.2" software.
We used RAPD for analysis of *S. aureus* and CoNS.

• ***Typing of coagulase-positive Staphylococci (S. aureus) by the PCR-RFLP method***
Coagulase encoded by the *coa* gene is produced by all strains of *S. aureus*. *Coa* gene polymorphism was used for the investigation of a PCR-based method for differentiation of *S. aureus* at the genetic level. 21 DNA isolates of *S. aureus* were typed by the PCR-RFLP method involving the amplification of the *coa* gene variable fragments (547-603-660-875bp) and the following digestion by the endonuclease *AluI*.

Results and discussion

Identification of methicillin resistance

The identification of methicillin resistance in *Staphylococci* and the collection of MRS strains were primarily based on the results of the standard agar disk diffusion method with oxacillin disk. To exclude the heterogeneity of resistance, all strains were additionally tested on 2% and 5% NaCl containing Mueller-Hinton agar and incubated at 32°C and 35°C. The results were compared with those obtained by the standard method. The use of the salt-supplemented medium demonstrated a remarkable increase in the detection of MRS strains – 5.6% of MRS were additionally registered. No difference was found between the 2% and 5% NaCl media and the incubation regimes 32°C and 35°C [22].

It is known that methicillin-resistant strains have acquired an additional 76-kDa penicillin-binding protein, termed PBP2a, encoded by the *mec*A gene and exhibiting a low affinity for β-lactam antibiotics. The *mec*A gene is integrated in a specific site of the chromosome of *Staphylococci* and is not found in methicillin-susceptible strains. The amplification of the *mec*A gene in PCR is still one of the basic methods for methicillin susceptibility testing in *S. aureus* and CoNS. In PCR-based detection, heterogeneous strains can be also revealed by the presence of the *mec*A gene.

Altogether, 122 Staphylococcal cultures isolated were analysed for the presence of the *mec*A gene. Among them, there were 56 coagulase-positive *S. aureus* (48 MRSA and 8 MSSA) and 66 CoNS, including 45 strains of resistant *S. epidermidis sensu strictu*, 15 *S. haemolyticus*, 3 *S. hominis*, 1 *S. capitis* and 1 *S. warneri,* and 1 methicillin-susceptible *S. epidermidis* isolate.

According to PCR results, all phenotypically methicillin-resistant *S. aureus* and CoNS strains showed the presence of the 310-bp fragment of the *mec*A gene, thereby confirming methicillin resistance (the method's sensitivity = 100%). For 8 of the 9 control strains, respectively, phenotypically methicillin-susceptible 8 MSSA and 1 *S. epidermidis*, the PCR

response was negative – the *mec*A gene was absent. One methicillin-susceptible *S. epidermidis* isolate, exhibiting oxacillin MIC 1 μg/ml, proved to possess the *mec*A gene and should be recognized as methicillin-resistant. The discordance of PCR and disk diffusion methods could be caused by the heteroresistant nature of *Staphylococci* or by the absence of the *mec*A gene expression on the phenotype. Our results correlated well with the previous studies, where the detection of the *mec*A gene was shown as more reliable than the disk diffusion method [24].

Additional testing of the strain using the E-test and the Mini Api system (Latvian Infectology Center, Dr. Ruta Paberza) confirmed methicillin resistance.

Concurrently, the Slidex MRSA detection test was used for the evaluation of *S. aureus* strains. For comparison purposes, we used some CoNS cultures.

Altogether, 34 strains identified as *S. aureus* (18 MRSA and 2 MSSA), *S. haemolyticus* (four), *S. epidermidis* (eight), *S. hominis* (one) and *S. capitis* (one) were analysed by the Slidex MSRA detection kit. Despite the prescription of using that test for *S. aureus* alone, we evaluated the potential of the Slidex test to be used for other *Staphylococci*. For each strain, negative latex control was used.

After 2-3 min, the latex agglutination reaction was clearly observed in all MRSA and in all *S. haemolyticus* isolates. However, S. *haemolyticus* is a coagulase-negative *coccus*. The *S. epidermidis* and *S. hominis* strains showed a weak agglutination after 10-20 min with small particles, in contrast to *S. aureus* and *S. haemolyticus*. 1 strain of *S. capitis* showed negative results even after 20 min. Negative results were also observed in MSSA strains. All methicillin-resistant isolates were *mec*A-positive, including the *S. capitis* strain.

Thus, the sensitivity of the Slidex test for *S. aureus* was 100%. Therefore, the Slidex test is recommended as very reliable for the identification of the methicillin-resistant *S. aureus*, being easier to perform in routine laboratories than PCR. In the present study, the Slidex MRSA test was shown as very accurate for the detection of methicillin-resistant *S. haemolyticus*. Therefore, it can be suggested that the Slidex MRSA test and the related latex agglutination-based methicillin resistance detection kits may be used for CoNS. Additional testing by the agar screen plate or detection of the *mec*A gene by PCR may help in the case of intermediate latex test results.

Undoubtedly, at least two methods should be applied in parallel for detection of methicillin resistance [11]. *Mec*A-PCR can be especially recommended for the detection of the presence of the *mec*A gene in cultures, which would help also in revealing hetero-resistant colonies. Besides, we recommend the Slidex MRSA test for rapid identification of methicillin resistance.

Prevalence studies of MRS

Studies were carried out during a 6-year period. The results are summarized in Fig. 1. The prevalence of MRSA during a 6-year period in our hospital differed insignificantly and was comparatively low. It is known from the literature that the prevalence of MRSA varies markedly from country to country with very high levels (> 60% of *S. aureus* isolates) being reported from the Far East and around 50% in South Europe, but far lower levels from Scandinavian and other countries, i.e. < 1% [14].

So, we can evaluate the prevalence of methicillin-resistant *Staphylococci* in our hospital as low in comparison with other hospitals.

As concerns the MR CoNS, a tendency of methicillin-resistant strains to increase was documented. We are prone to conclude that it may be associated with the implementation of more precise diagnostic methods.

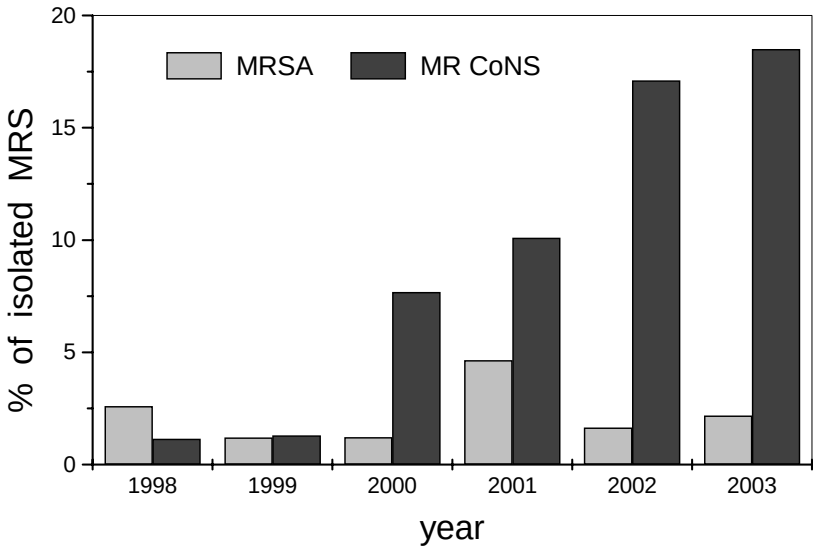

Fig. 1 Prevalence of MRSA and MR CoNS during 6 years

Molecular typing of MRS

As mentioned previously, three methods have been used for molecular typing of MRS.

Randomly Amplified DNA polymorphism (RAPD) analysis was applied to 24 MRSA and 10 coagulase-negative *Staphylococci*. 24 MRSA strains were grouped in 3 main groups (the largest one included 8 strains) with the pattern similarity 70-100% and were further analysed for epidemiological links. A possible transmission was confirmed for two cases. Coagulase-negative *Staphylococci* had more diverse patterns, and only one group was formed.

The discriminatory power of RAPD was compared to that of the PFGE method. It was found to be lower, however, RAPD was good to show the similarity or, on the opposite, the diversity of strains. Therefore, it could be applied for preliminary screening of MRSA before PFGE (Fig. 2).

The PFGE method was used for typing of *S. haemolyticus* resistant strains isolated from 10 bacteremia patients.

Restriction results were available for 9 strains (Fig. 3). Restriction patterns were scanned and compared using the "GelCompar4.2" computer software. Two clusters of completely identical strains were found. Each cluster consisted of two strains: one cluster was formed by the strains 78/82 and 1063/1146 (Fig. 3, lanes 2 and 7), another – by the strains 379/445 and 91/115 (lanes 3 and 4). The remaining strains were heterogeneous. However, no epidemiological links were identified between the patients corresponding to isolates, since they were admitted at hospital in different time and in various units.

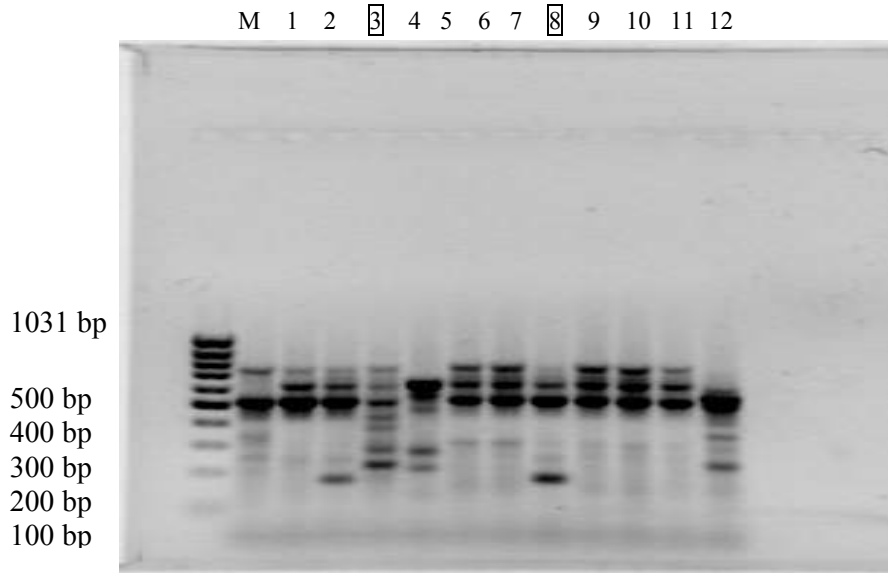

Fig. 2 RAPD analysis example. M – molecular weight marker, lanes 1-12 – patterns of MRSA strains

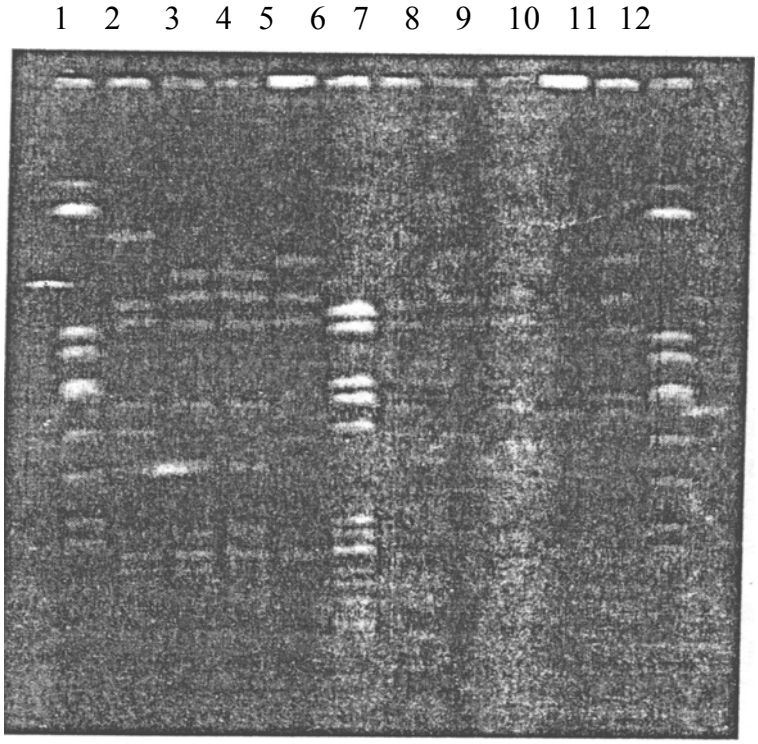

Fig. 3 *SmaI* macrorestriction patterns of *S. haemolyticus* separated by PFGE. Lanes 1 and 12 – standard MRSA strain NCTC8325; lanes 2-11 – S. *haemolyticus* strains

PCR products of the *coa* gene were obtained from 21 DNA isolates of *S. aureus* and subsequently digested with *AluI* (MBI Fermentas). Digestion products were analysed in 6% PAAG (Fig. 2). 16 (80%) of 21 isolates had similar patterns, 2 and 1 isolates were different from this pattern. Digestion had a failure for 3 isolates; it showed a complete PCR fragment. A high percent of the pattern similarity was recovered in our isolates; therefore, this method cannot be applied for *S. aureus* strain typing in Latvia. Similar results were obtained at the Robert Koch Institute (RKI), Germany (personal communication).

Molecular typing is necessary not only for epidemiological purposes. Although antimicrobial resistance is often described as a progressive process associated with the use of antimicrobials, actually, it may be a spontaneous process, which results from genetic mutations and the acquisition of an exogenous genetic material. So, the study of bacterial genotypes may help in predicting of antimicrobial resistance [5].
Our studies in the field are continuing.

Antimicrobial susceptibility of MRS

The antimicrobial susceptibility of *Staphylococci* was tested on a regular basis (Fig. 4, Fig. 5).

There was no tendency for changing susceptibility during the period under study for any of the tested agents. Except the β-lactam antibiotics, the highest resistance was registered for erythromycin, followed by ciprofloxacin, clindamycin, gentamicin. No strains were found susceptible to all 5 tested antibacterials.

All the MRS isolates were susceptible to vancomycin. Detailed results about the susceptibility to antibacterials of different species are published [24].

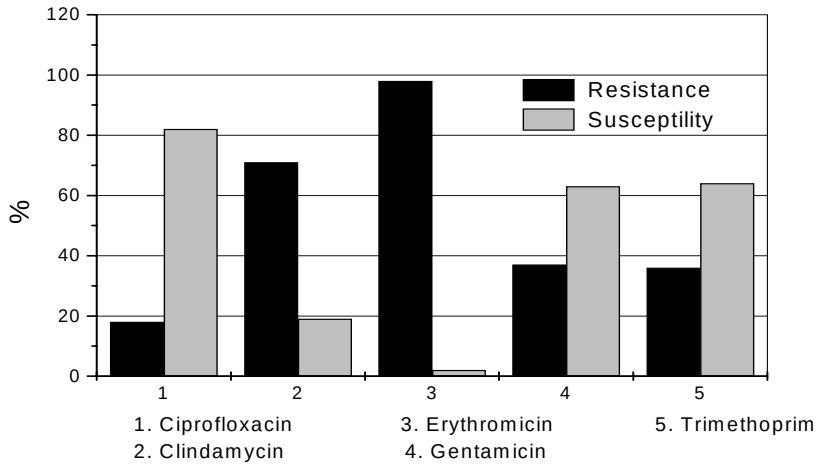

Fig. 4 Methicillin resistant *Staphylococcus aureus.* Susceptibility to antimicrobials

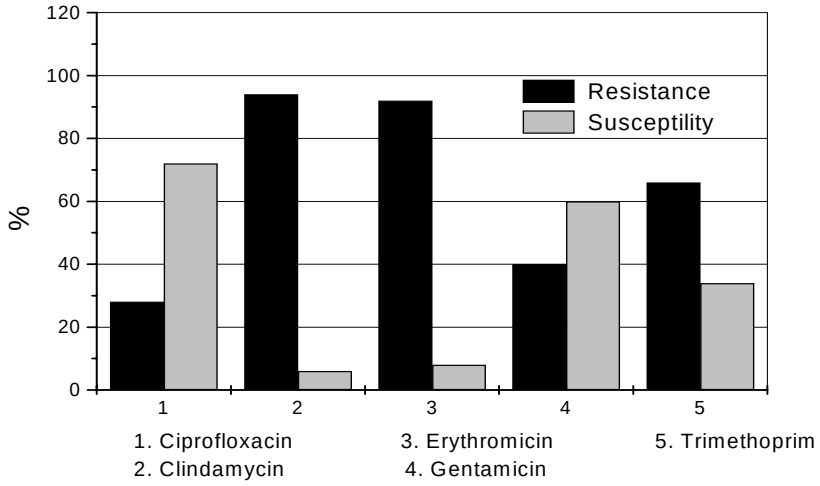

Fig. 5 Methicillin resistant coagulase negative *Staphylococci.* Susceptibility to antimicrobials

Conclusion

Methicillin resistance in *Staphylococci* was detected by phenotypical tests and confirmed by the *mec*A PCR for all the strains and the Slidex MRSA test. Undoubtedly, at least 2 methods are necessary for detection of methicillin resistance. From molecular typing methods, PFGE occurred to be most reliable. Over the monitored 6-year period, the prevalence of MRSA strains did not differ significantly and was comparatively low in our hospital. The MR CoNS prevalence has increased during the last 6 years. All isolated MRS strains were multiresistant. Vancomycin resistance was not registered.

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