Experiment	**Pathogenic Staphylococci of the Nose ?** **Molecular Diagnostics and Importance of Antibiotic Resistances**
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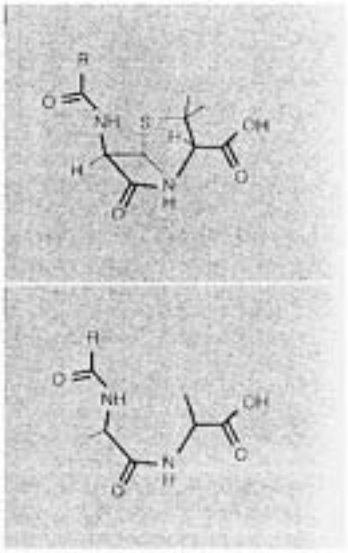
Reading	Chapters in BBOM 9th: **3.7, 18.7, 18.8, 18.12**, 19.2, 21.1, **21.3**, , 22.7, 22.8 BBOM 9th: Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, Prentice Hall, 1999. Corresponding chapters in the10th Edition: **4-8, 20.7, 20.8, 20.12,** 21.2**,** 24.1, **24.3,** 25.7, 25.8
Objectives	To determine **antibiotic resistance** in an important human pathogen. To demonstrate the induction of the **penicillin-binding protein PBP2a** and **methicillin resistance**. Identification of antibiotic resistance mechanisms in clinical isolates.
Background	*Staphylococcus aureus* is a normal, permanent or transient inhabitant of skin and mucous membranes in 10 to 30 % of healthy people. *S. aureus* has though, a pathogenic potential, and can produce **toxins** and **virulence factors**, causing superficial infections, food poisoning, and more serious diseases, such as endocarditis, osteomyelitis, bacteriaemia, and toxic shock. Staphylococci have the ability to develop relatively easily **resistance to antibiotics**, either by **mutation** or by **acquisition of resistance determinants**. Especially methicillin-resistant staphylococci are feared, because, besides their intrinsic resistance to all **beta-lactams** and derivatives, they have the tendency to acquire resistances to multiple additional, not related antibiotics. Due to the high antibiotic use and pressure in hospitals, some strains of *S. aureus* have become resistant to almost all commonly used antibiotics. It is therefore of importance to determine if an *S. aureus* isolate from a patient carries the methicillin resistance determinant, in order to take special precautions to prevent the spread of a potential methicillin-resistant *S. aureus* (MRSA) to other patients. Methicillin was the first **semi synthetic penicillinase-resistant beta-lactam**. Staphylococci that are resistant to methicillin are also resistant to penicillin and to all other penicillinase-resistant derivatives of beta-lactams, including cephalosporins and carbapenems. The **resistance is intrinsic**; this means that the antibiotic is not destroyed, but that it has no effect any more on the methicillin-resistant *S. aureus*. Resistance is due to the acquisition of the ***mecA* gene**, which codes for an additional penicillin-binding protein, called PBP2a. This PBP2a has a much lower affinity to beta-lactams than the cells own PBPs, and still functions at concentrations of methicillin, which inactivate the normal PBPs. PBPs catalyze the crosslinking of the peptide moiety of the **peptidoglycan**. Beta-lactams inhibit the **transpeptidase activity** of the PBPs and thus the crosslinking of the cell wall. This is lethal for the cells, because a strong cell wall is needed to maintain the shape of the bacteria and to confer strength against the internal osmotic pressure.



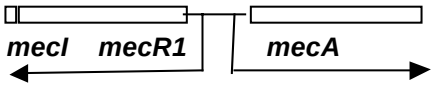
Penicillin mimicks the structure of the terminal D-Ala-D-Ala of the peptidoglycan precursor, the substrate of the PBPs. Penicillin binds to the active site of the transpeptidase domain of the PBPs and inactivates their function.

From: http://www.systbot.uni-tuebingen.de//penicillin/chemie.htmls

Background on penicillin action

Steps involved in bacterial **cell wall synthesis** are suitable targets for antibiotics. **Penicillin** inhibits cell wall biosynthesis and leads to lysis and death. Penicillin acts by preventing the **cross-linking of the peptide chains** of the bacterial **peptidoglycan**. The enzymes involved in these last steps of cell wall synthesis are called **penicillin-binding proteins (PBP).**

Regulation of PBP2a production



The *mecA* operon

The **repressor** MecI prevents transcription of the *mecA* gene and of *mecR1-mecI* in absence of an **inducer**. In presence of an inducer the transmembrane sensor-transducer MecR1 inactivates the repressor MecI and allows so transcription of *mecA* and thus PBP2a production.

Methicillin–resistance in staphylococci containing the *mecI-mecR1-mecA* **operon** is strongly repressed. Induction of PBP2a by methicillin or oxacillin is very slow. Such strains may appear to be susceptible to methicillin, although they carry the methicillin resistance determinant. Cefoxitin is a better inducer than oxacillin or methicillin for PBP2a. Mutants with constitutive PBP2a production have either lost the repressor or have a mutated operator region, which does not allow binding of the repressor any more.

Clinical isolates of *S. aureus* often harbour penicillinases, which confer resistance to penicillins, but not to methicillin. Due to the very similar sequences of the penicillinase regulatory systems with that of PBP2a, the penicillinase repressor BlaI controls also *mecA* transcription.

The expression of the methicillin resistance is heterogenous

The levels of methicillin resistance vary from methicillin-resistant *S. aureus* (MRSA) to MRSA. MRSA are sometimes difficult to distinguish phenotypically from susceptible *S. aureus* (MSSA). Some strains despite

	of carrying the resistance gene *mecA,* do not express the resistance. However, upon exposure to beta-lactams, they segregate highly resistant mutants. These highly resistant mutants are responsible for the failure of beta-lactam therapy of MRSA infections. For the clinician it is therefore very important to know if a strain carries the *mecA* gene, even if the resistance is only weakly expressed, in order to prescribe the correct antibiotic therapy. **Definition of resistance: When are bacteria resistant to antibiotics?** Clinically — therapy resistance Microbiologically — minimal inhibitory concentration (MIC) Mutation — difference in resistance between parent and mutant Genetically — acquired resistance determinant Biochemically — resistance mechanism Inherent — genus- or species-specific resistance Resistance in bacteria can be determined: • Phenotypically, by measuring the **minimal inhibitory concentration** (**MIC**) of the antibiotic. By comparing resistance between parent and mutant. • Genetically, by showing the presence of a **resistance determinant sequence** or a particular mutation. • Biochemically, by demonstrating the presence of **a resistance mechanism**, or of the reaction leading to resistance.
Literature	Archer G.: *Staphylococcus aureus*: a well-armed pathogen. Clin Infect Dis. 1998, 26 : 1179-81. Review. Chambers HF.: Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev. 1997, 10: 781-91. Review.
www. Links	
Practical work	You will learn different methods to determine antibiotic resistance in bacteria. You will determine the production of PBP2a in induced cells of a methicillin-resistant *Staphylococcus aureus*. You will test if strains are producing a penicillinase. You will determine the antibiotic resistance in two unknown clinical isolates and from the results deduce the resistance mechanisms. The methods used are: - Disk diffusion method - E-Test method - Microbroth dilution - Nitrocefintest (penicillinase) - PBP2a agglutination

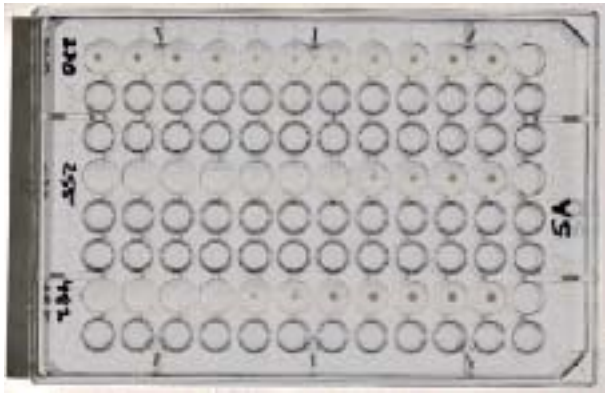
Disk diffusion

Paper disks containing defined amounts of antibiotics are placed on a lawn of bacteria. The size of the inhibition zones correlate with the antibiotic resistance levels of the bacteria.

Etest method

The E-strip contains a defined gradient of an antibiotic. It is placed on a lawn of bacteria. The minimal inhibitory concentration of the antibiotic can be read at the intersection of growth with the E-strip.

Microbroth dilution

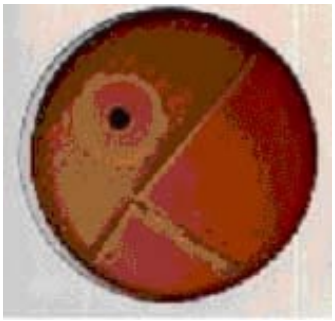


A suspension of 10^4 bacteria is added to decreasing twofold dilutions of an antibiotic. The first well with no growth corresponds to the minimal inhibitory concentration of the antibiotic.

	Latex agglutination  Latex beads coated with anti-PBP2a antibodies agglutinate with the PBP2a on MRSA to form a precipitate. Left to right: MRSA, MRSA control for self-agglutination, MSSA control, MSSA control for selfagglutination. **Nitrocefintest** Penicillinases cleave nitrocefin to produce a deep red colour.
Material and Experimental Protocols	You will characterize clinical MRSA isolates. As control you will use a susceptible *S. aureus* strain (MSSA) and an isogenic methicillin–resistant strain (MRSA). You will determine: • the resistance of the strains to different antibiotics • the MIC of oxacillin • the presence or absence of PBP2a • the presence or absence of a penicillinase You obtain the strains to be tested on sheep blood agar plates. By holding the plates against the light you can see if they produce hemolysins. (Seen as a clear zone around the colonies). **Day one:** Disk diffusion assay E-Test Induction of *mecA* by cefoxitin Microbroth dilution **1. Preparing a working suspension of the strains to be tested** Pick up 3 to 5 colonies with a cotton tip applicator and suspend them into a tube containing sterile saline. Adjust the turbidity of the suspension to 0.5 McFarland (by comparing the turbidity to a reference tube containing the 0.5 McFarland standard). This working dilution contains approximately 10^8 bacteria/ml. Keep this solution for the following experiments 2, 3, and 4. **2. Resistance tests: Disk diffusion assay/E-test** Dip a fresh cotton tip applicator in the working suspension and swab two Müller-Hinton plates evenly to cover the entire surface. Change the angle by 120 ° and repeat this two times. The surface of the agar plates is now inoculated with a more or less homogenous layer of bacteria. **E-test:** Applicate an oxacillin Estrip in the middle of one plate with a sterile forceps. **Disk diffusion:** On the other plate stamp with an applicator paper disks impregnated with different antibiotics.

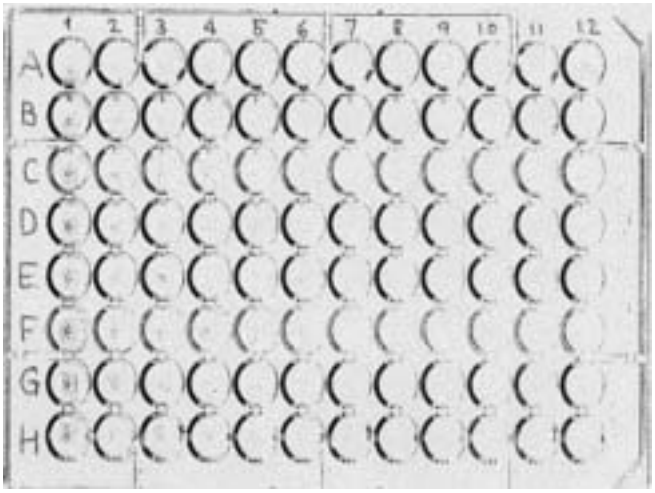
3. Induction of *mecA* by cefoxitin

With the cotton tip applicator inoculate half of a sheep blood agar plate with the clinical isolate. Fractionate the bacteria with a plastic loop over the remaining half plate as will be demonstrated. Add a cefoxitin paper disk on the first fraction (cefoxitin is a penicillin-derivative, that can act as an inducer of *mecA*). This plate will be used in the next course to determine PBP2a by agglutination.



4. MIC determination by microbroth dilution

The microbroth dilution allows determining MIC of antibiotics in broth. The microtiter plate contains a series of 12 wells in a row. Use one row for each strain.



1. Pipet 50 ml of broth into the wells 2 to 12 of the microtiter plate.
2. Add 50 ml of the oxacillin stock solution to the wells 1 and 2.
3. Make a geometric dilution of the antibiotic with the factor two: For this purpose: take 50 ml from well 2 and add it to well 3, mix by pipetting up and down. Continue this procedure up to well 10. Well 10 contains now 100 ml, remove and discard 50 ml from it. Well 11 is the growth control with no antibiotic. Well 12 is the control for the sterility of the broth.
4. Dilute the working suspension of the clinical isolate 100 fold by adding 20 ml of the working dilution into 2 ml of broth.
5. Add 50 ml of the diluted working suspension into the wells 1 to 11. Cover the plate with a sticking plastic sheet and incubate over night at 37 °C. In the next course, the growth in each well can be read. The MIC of oxacillin corresponds to the lowest concentration of the antibiotic where no growth occurs.

Day 2:
PBP2a agglutination
Reading of the MICs and inhibition zones
Nitrocefintest
Interpretation of the results

2. Agar dilution, E-test
Measure the inhibition zones around the paper disks and determine if the strains are resistant or susceptible against the antibiotic.
Determine the minimal inhibitory concentration of oxacillin that can be read at the intersection of the Estrip. Inspect the inhibition zone carefully for colonies appearing in the inhibition zone.

3. PBP2a Agglutination
You will make agglutinations with latex beads loaded with anti-PBP2a antibodies to demonstrate the presence of PBP2a in the clinical *S. aureus* isolates. To be certain that PBP2a is expressed, the clinical MRSA isolate to be tested have been induced by cefoxitin. For this purpose take bacteria that grow close to the inhibition zone of cefoxitin(induced culture).
As negative control use strain MSSA, as postive control use strain MRSA.

Extraction of PBP2a
For each culture probe:
- Add 4 drops of **Extraction-Reagent 1** in a 1.5 ml Eppendorf tube
- Suspend a plastic loop full of induced bacteria in this tube
- Heat the suspension for 3 min to 95 °C and let it cool again to room temperature
- Add 1 drop of **Extraction-Reagent 2**, mix well
- Centrifuge for 5 min at 4500 rpm in an Eppendorf centrifuge

Latex agglutination
- For each probe prepare a **test card** with a ring for the probe and one ring for the control.
- Add in the first ring one drop of **Sensitized-Latex** and 50 ml of the supernatant ; mix with the plastic stick.
- Add one drop of **Control-Latex** in the second ring and 50 ml of the supernatant; mix with the plastic stick. (= test for selfagglutination)
- Rotate the test-card for 3 min by hand and observe if you see agglutination.

Interpretation
- No agglutination with Sensitized-Latex and Control-Latex: PBP2a negative.
- Agglutination with Sensitized-Latex, no agglutination with Control Latex: PBP2 a positive.
- Agglutination with Control-Latex: undefined, repeat reaction.

4. Microbroth dilution
Read the minimal inhibitory concentration of oxacillin.

5. Nitrocefintest
Take a loopful of induced bacteria and mix it with nitrocefin. The colur changes from yellow to red in presence of penicillinases.

Laboratory Rules & Precautions	*Staphylococcus aureus* is a normal inhabitant of skin and mucous membranes in 10 to 30 % of healthy people. It has, though, a pathogenic potential and can produce toxins and virulence factors. You have to wear a lab coat. No eating or drinking in the lab. Care must be taken not to contaminate the environment. Aerosol formation has to be prevented. All waste must be sterilized before disposal. Hands must be washed and disinfected and surfaces wiped with 70 % ethanol after completion of the experiments.
Experiences gained	• To learn different methods of antibiotic resistance determination. • To see the difference between induced and uninduced cultures.
Timing	1st day 1.5 h, 2nd day 1 h
Reporting	Report: - Inhibition zones of the different antibiotics: interpret if the strains are resistant or susceptible - Oxacillin MICs by Etest : do you see single colonies in the inhibition zone? - Oxacillin MIC by microbroth dilution - Agglutination results with the two clinical isolates - Interpret and explain the results.
Questions to be answered	• How can methicillin resistant bacteria become constitutive producers of PBP2a? • Does penicillin act on resting cells? • Compare the advantages and disadvantages of genetic versus phenotypic determination of the resistance.