Faculty of Medicine
University of Rijeka

TransMedRI Workshop (*hands-on*)

# Molecular Methods in Microbiology and Epidemiology

June 12 – 15, 2012

## Department of Microbiology

Faculty of Medicine, University of Rijeka, B. Branchetta 20, Rijeka, Croatia

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Dear Collegues,

**You are cordially invited to attend the lectures and participate in hands-on workshop!**

Workshop objective is to provide the participants with the information about new developments in the fields of microbiology and give them an overview of the molecular methods in microbiology and epidemiology.

Practical part of the workshop is going to provide the participants with hands-on experience of molecular typing of *Klebsiella pneumoniae* by PFGE, and the basic information about the analysis of PFGE results by GelCompar II software.

Target audience are medical doctors, laboratory engineers, and other professionals that work in the molecular microbiological laboratories. There is no registration fee. While the theoretical part of the workshop is open to everyone, number of the participants of the practical part is limited to 15.

Organizers:

Maja Abram, Marina Šantić, Mihaela Matovina, Vanja Vasiljev Marchesi, Tomislav Rukavina

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***Workshop on***

***Molecular Methods in Microbiology and Epidemiology***

***Rijeka, June 12th – 15th, 2012***

***University of Rijeka, Faculty of Medicine***

***Department of Microbiology***

# Workshop Program

Day I – Tuesday June 12th, Lecture Hall

| | |
|---|---|
| 11:00 – 12:00 | **Anders Sjostedt**, University of Umeå, Sweden |
| | *Advice and regulations regarding BSL3 facilities* |
| 12:00 – 12:30 | Coffee break |
| 12:30 – 13:30 | **Evelyn Stelzl,** Medical University of Graz, Austria |
| | *Trends in routine molecular diagnostics* |
| 13:30 – 14:00 | **Mihaela Matovina**, Faculty of Medicine, University of Rijeka |
| | *Molecular typing by Pulsed Field Gel Electrophoresis (PFGE) - introduction* |
| 14:00 – 15:00 | Lunch |

Day II – Wednesday June 13th, Department of Microbiology

**Hands-on laboratory work: PFGE for *Klebsiella pneumoniae* genotyping** (Mihaela Matovina, Vanja Vasiljev Marchesi)

| | |
|---|---|
| 09:00 – 12:00 | Bacterial cultures preparation |
| 12:00 – 13:00 | Lunch |
| 13:00 – 17:00 | Agarose plugs preparation |
| | Incubation of plugs in lysis buffer 1 |
| | Washing |
| | Incubation of plugs in lysis buffer 2 |

Day III – Thursday June 14th, Department of Microbiology

**Hands-on laboratory work: PFGE for *Klebsiella pneumoniae* genotyping** (Mihaela Matovina, Vanja Vasiljev Marchesi)

| | |
|---|---|
| 09:00 – 13:00 | Washing |
| | Preparation of samples for restriction enzyme digestion |
| | Restriction enzyme digestion |
| 13:00 – 15:00 | Lunch |
| 15:00 – 17:00 | Sample loading |
| | Gel preparation |
| | Electrophoresis |

Day IV – Friday June 15th, Lecture Hall

| | |
|---|---|
| 09:30 – 09:50 | **Suzana Bukovski,** University Hospital for Infectious diseases "Dr. Fran Mihaljević", Zagreb |
| | *When, why and how to apply molecular diagnostics of invasive bacterial infections* |
| 09:50 – 10:10 | **Ana Budimir**, Clinical Hospital Center and School of Medicine, University of Zagreb |
| | *Molecular typing methods for bacterial pathogens* |
| 10:10 – 10:30 | **Vera Katalinić Janković,** Croatian National Mycobacteria Reference Laboratory, Zagreb |
| | *Molecular tools in diagnosis and TB control* |
| 10:30 – 11:00 | Coffee break |
| 11:00 – 11:20 | **Ivana Goić Barišić,** Clinical Department of Microbiology and Parasitology, Split University Hospital and Faculty of Medicine, University of Split |
| | *Molecular epidemiological investigation of carbapenem-resistant Acinetobacter baumannii isolates from Split University Hospital during ten years period* |
| 11:20 – 11:40 | **Mihaela Matovina,** Faculty of Medicine, University of Rijeka |
| | *Genotyping of Pseudomonas aeruginosa by PFGE* |
| 11:40 – 12:00 | Discussion |
| 12:00 – 14:00 | Lunch |

Department of Microbiology

**Hands-on laboratory work: PFGE for *Klebsiella pneumoniae* genotyping** (Mihaela Matovina, Vanja Vasiljev Marchesi)

| | |
|---|---|
| 14:00 – 16:00 | Gel staining and documentation |
| | Analysis of PFGE results |
| | GelCompar II software demonstration |
| 16:00 – 16:15 | Closing remarks |

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# Abstracts and Laboratory Protocols

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## Advice and regulations regarding BSL3 facilities

**Anders Sjostedt**

Molecular Infection Medicine Sweden (MIMS) and Department of Clinical Microbiology

**University of Umeå, Sweden**

The presentation will discuss regulations regarding BSL3 facilities and also provide advice derived from more than 20 years of work with such facilities. Specifically, the presentation will deal with general advice regarding construction of a BSL3 facility and safety and security considerations in this regard. In addition, the handling of emergency responses following internal or external incidents will be discussed. Moreover, guidelines for exercises and training will be provided as well as suggestions for how an emergency response team should be functioning.

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# Trends in routine molecular diagnostics

**Evelyn Stelzl**

Research Unit Molecular Diagnostics and Molecular Diagnostics Laboratory

**Medical University of Graz, Austria**

In the past, molecular methods for detection of nucleic acids included several manual steps. Due to the labor-intensive procedure, they were error-prone with a high risk of contamination.

In the present, improved molecular diagnostics instrumentation automates most of the assay steps allowing for high-volume testing. Currently, there are several platforms for automated nucleic acid extraction on the market. Furthermore, new molecular assays with the majority of them being IVD/CE-labeled are regularly introduced to the market by several manufacturers. Consequently, the risk for contamination has been significantly reduced.

For the majority of assays, there is still a separation between the extraction and amplification/detection parts. In the future, this may be overcome by a combined extraction and real-time PCR procedure.

Sequencing is another increasingly used technology in the molecular diagnostics laboratory. Currently, the conventional Sanger technique is commonly used; however, this may be replaced by one of the so-called “next generation sequencing” technologies in future.

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# Molecular typing by Pulsed Field Gel Electrophoresis (PFGE)

**Mihaela Matovina**

Department of Microbiology and Parasitology

**Faculty of Medicine, University of Rijeka, Croatia**

Despite large number of methods developed for bacterial genotyping pulsed field gel electrophoresis (PFGE) is still considered a "gold standard" of molecular typing methods for a variety of microorganisms, especially in the clinical setting. PFGE is the technique for separating large DNA molecules (> 40-50 kb), which cannot be separated by standard gel electrophoresis. To perform typing by PFGE, bacteria are first mixed with agarose and poured into moulds to imbed the cells in the agarose matrix. This is done to prevent shearing of bacterial DNA during isolation. Imbeded cells are treated with lysozyme (gram positive bacteria) and proteinase K, which results in DNA imbeded in the agarose matrix. Agarose-imbeded DNA is cut with "rare cutter" restriction enzyme (restriction enzymes with infrequent recognition sites in bacterial genomes), and fragments are separated by PFGE, resulting in the characteristic pattern of bends for each strain. Gels are stained with ethidium bromide, and photographed under UV light. Photographs are analyzed by computer with Gelcompar II (Applied Maths NV, Belgium) or similar software, and dendogram, which shows genotypic relation of the strains, is constructed.

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# When, why and how to apply molecular diagnostics of invasive bacterial infections

**Suzana Bukovski**

Department for Clinical Microbiology, Division of Bacteriology and Hospital Infections

**University Hospital for Infectious Diseases "Dr. Fran Mihaljević", Zagreb, Croatia**

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# Molecular typing methods for bacterial pathogens

**Ana Budimir**

Department of Clinical Microbiology and Molecular Microbiology

**Clinical Hospital Center and School of Medicine, University of Zagreb, Croatia**

Molecular strain typing has become an essential tool for the analysis of bacterial pathogens obtained during investigations of epidemiological outbreaks, laboratory contamination and recurrent infection.

Since microorganisms were first isolated and grown in pure culture, microbiology laboratories have needed to characterize isolates so that they can be differentiated from one another. The development of molecular biology in the 1980s contributed a set of powerful new tools for microbiologists to detect the smallest variations within microbial species and even within individual strains.

In fact, the technology has progressed far beyond the level needed by most routine laboratories. Distinguishing between different strains of the same species – typing – is more likely to be of value in a research laboratory or in more specialized fields, such as epidemiology.

## Typing

There are a number of reasons why it may be necessary to characterize a microbial isolate beyond species level and determine its sub-species, strain, or even sub-strain. For example:

To relate different cases during an outbreak of infectious disease

To establish an association between an outbreak of food poisoning and a specific food

To study variations in the pathogenicity, virulence and antibiotic resistance of individual strains within a species

To trace the source of contaminants within processing of specimens....

Phenotypic methods, as phage typing, have been used for many years to type certain bacterial pathogens. Other traditional typing systems include biotyping (based on detailed biochemical characteristics), bacteriocin typing and protein typing.

These are all phenotypic typing techniques relying on expressed characteristics and some have been developed into sophisticated systems with a range of applications. For example, rapid, semi-automated Salmonella serotyping systems have been developed and typing of extracted cell proteins can be carried out using mass spectrometry techniques such as MALDI-TOF (Matrix Assisted Laser Desorption Ionisation - Time of Flight). Analysis of cellular fatty acid methyl esters (FAMEs) by gas chromatography has also been used to type bacteria successfully. Some of these techniques have been developed into commercial typing systems that include profile databases and software to assist with accurate typing.

**Genotyping**

Ideally, sequencing or the entire genome, would provide definitive typing of microorganisms. Deffinitively more practical, if slightly less discriminating, methods for analysing DNA extracted from microbial cells – often termed 'DNA fingerprinting' techniques – have been developed and some of the more widely used methods are outlined below.

1. Multilocus sequence typing (MLST) – sequencing 400-500 base pair fragments of DNA at seven different conserved genes allows small variations within a species to be detected. Quite time consuming and costly, but can be highly discriminatory if the genes are correctly chosen.

2. Pulsed-field gel electrophoresis (PFGE) – a technique that allows the electrophoretic separation of low numbers of large DNA restriction fragments produced using restriction enzymes to generate a highly discriminatory genetic fingerprint.

3. Ribotyping – this technique relies on the relative stability of the 16S and 23S rRNA genes coding for ribosomal RNA. The genes are cut using restriction enzymes and resulting DNA fragments separated by electrophoresis.

4. Repetitive sequence-based PCR (rep-PCR) – bacterial and fungal genomes contain numerous non-coding, repetitive DNA sequences separating longer, single copy, sequences and their arrangement varies between strains.

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# Molecular tools in diagnosis and TB control

**Vera Katalinić Janković**

Croatian National Institute of Public Health and

**Croatian National Mycobacteria Reference Laboratory, Zagreb, Croatia**

Treatment of tuberculosis patients and control of M. tuberculosis infection spreading begins with an accurate, reliable and prompt diagnosis. The progress in TB bacteriology was always slow and methods applied were time-consuming. Nevertheless, developments in molecular biology methods enabled the introduction of rapid methods into TB diagnostics and, consequently, sped up the diagnostic algorithm. Simultaneously, the network of laboratories as part of the national TB control programs had to grow stronger as molecular methods can only be introduced into well-equipped controlled labs with trained and experienced staff. A modern TB lab is expected to communicate smear results within 24 hours from sample delivery, to detect and identify 80% M. tuberculosis cases in less than two weeks, and to complete antitubercular sensitivity testing for 75% positive M. tuberculosis samples in three weeks. Amplification technique-based tests, such as polymerase chain reaction (PCR), are used today in mycobacteriology on different levels. PCR is applied for agent detection directly from sample, identification, determination of molecular basis of resistance and genotyping of isolated M. tuberculosis strains. Genotyping methods contributed to a better understanding of TB epidemiology and laboratory contamination analytics. Still, these methods have not replaced the gold standard of microscopy and cultivation, but merely perfected it. They are used for detection of M. tuberculosis directly from clinical samples in combination with cultivation methods as their sensitivity is lower than that of the latter. Diagnostic algorithm for TB labs is still in quest for so called POC (Point Of Care) testing which would be user-friendly, reliable, cheap, as well as applicable at the bedside.

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# Molecular epidemiological investigation of carbapenem-resistant *Acinetobacter baumannii* isolates from Split University Hospital during ten years period

**Ivana Goić Barišić, Marija Tonkić, Anita Novak**

Clinical Department of Microbiology and Parasitology

**Split University Hospital and Faculty of Medicine, University of Split, Croatia**

*Acinetobacter baumannii* is an important opportunistic pathogen that is rapidly evolving towards multidrug resistance and is involved in nosocomial infections, especially in intensive-care units. Nosocomial *A. baumannii* infections mostly include ventilator-associated pneumonia, bloodstream infection, and wound and urinary catheter infections in critically ill patients. *A. baumannii* can survive on fingertips and inanimate objects such as glass, plastic and other enviromental surfaces, even after exposure to dry conditions durig extended periods of time. The survival of *A. baumannii* has also been attributed to resistance of this microorganism to antimicrobial drugs and desiccation. Until the 1970s, most isolates were susceptible to a wide range of antibiotics. The emergence in resistance trends arises in ability to 'switch' its genomic structure, combined with variable gene expression and presence of mobile elements, that probably explains the unmatched speed at which *A. baumannii* can respond to selection pressure from antimicrobial agents. Many outbreak strains belong to one of three worldwide lineages, known originally as European clones I, II and III. These correspond to sequence groups 2, 1 and 3, respectively, each of which includes a number of different genotypes defined by pulsed-field gel electrophoresis (PFGE). To investigate the molecular epidemiology of *A. baumannii*, a variety of typing systems have been developed, including ribotyping, genome analysis with selective amplified fragment length polymorphisms, randomly amplified polymorphic DNA analysis, infrequent-restriction-site PCR, and pulsed-field gel electrophoresis (PFGE). PFGE restriction analysis of chromosomal bacterial DNA has been used with exellent results in epidemiological studies of numerous *A. baumannii* outbreaks, and is currently regarded as the reference standard for epidemiological typing.

Carbapenems (especially imipenem and meropenem) have been a mainstay of treatment for acinetobacter infections for the past decade. However, increasing number of clinical isolates of *A. baumannii* resistant to carbapenems are now being reported worldwide, reaching levels of ≥ 90% in some centres. These levels are much higher in southern European countries like Spain, Italy, Greece and lately Croatia. At Split

University Hospital, the first carbapenem (meropenem) resistant strain of *A. baumannii* was isolated in 2002 from urine sample at Intensive Care Unit. Over a period lasting seven years carbapenem resistance in clinical isolates of *A. baumannii* at Split University Hospital did not exceed 30 % for meropenem and 10 % for imipenem. Molecular epidemiological investigation on more than hundred non repetitive patient isolates of *A. baumannii* collected between 2002 and 2007 at Split University Hospital confirmed the presence of European clone I as dominant clone by PFGE and PCR-based sequence typing. Molecular characterisation of reduced susceptibility to carbapenems discovered insertion sequence ISAba1 upstream of bla OXA 51/69 like gene in majority strains. Isolates from Split University Hospital contained the newer OXA-107 enzyme, closely related to OXA-69, with an amino-acid change at position 167 that replaces leucine with valine, and described in *A. baumannii* isolates only from Poland and Slovenia.

In the beginning of 2009, a 51 year-old female was transferred to the ICU of Split University Hospital, Croatia, following brain surgery at the General Hospital Mostar, Bosnia and Herzegovina. During the hospitalization in General Hospital Mostar, *Acinetobacter* spp. resistant to all tested antibiotics was isolated from a bronchial aspirate. Following the transfer of patient to Neurosurgery ICU Split University Hospital, multidrug-resistant *Acinetobacter baumannii* was isolated from bronchial lavage, blood culture and cerebrospinal fluid. During the next 12 months more than 50 similar isolates of *A. baumannii* with the same multidrug resistance pattern (no inhibition zone around imipenem or meropenem discs) were obtained from different departments and ICUs at Split University Hospital. All collected isolates were identified by ATB 32GN and Vitek 2 systems (bioMerieux, Marcy l' Etoile, Francuska). PFGE following macrorestriction of genomic DNA with ApaI confirmed identify with origin isolate and emphasized dissemination of a new clone, European clone II during 2009 inside Split University Hospital. Molecular investigation for presence of genes encoding carbapenem resistance was detected by multiplex PCR and confirmed the presence of OXA-90 gene (a variant of OXA-51/66), gene encoding OXA-72 (a variant of OXA-40 like family), and VIM-type gene cassette for metallo-enzyme. The new clone of *A. baumannii*, so called „mostar clone“, has become dominant at Split University Hospital and give rise to dramatic increase in carbapenem resistance (89,6%) as compared with the previous years accordind data of the Croatian Academy of Medical Sciences.

Prevention of infections requires knowledge about epidemiology of infections. This epidemiology can be straightforward when sources and reservoirs have been identified and when it is easy to differentiate between sporadic and epidemic cases of infection. However, the development of molecular typing methods has provided clinical microbiologists with powerful tools to explore the spidemiology of infections and make possible implementation of control measures.

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# Genotyping of *Pseudomonas aeruginosa* by PFGE

**Mihaela Matovina**

Department of Microbiology and Parasitology

**Faculty of Medicine, University of Rijeka, Croatia**

*Pseudomonas aeruginosa* is an important human pathogen, characterized by environmental versatility, ability to cause a wide range of human infections in susceptible individuals, and extensive antibiotic resistance. Molecular typing of P. aeruginosa is an important tool in determining the spread of multi resistant strains of bacteria in the hospital environment. There are different methods available for the molecular typing of *P. aeruginosa* today, however, pulse field gel electrophoresis (PFGE) is still considered the "gold standard". We analyzed 56 clinical isolates of carbapenem resistant *P. aeruginosa* collected from different departments of University Hospital Rijeka, Croatia by PFGE, after macrorestriction with SpeI restriction enzyme. Acquired data was analyzed by BioNumerics GelCompar II® software (Applied Maths NV, Belgium). Typeability of isolates was 98.2%, only 1 out of 56 samples could not be analyzed. Gel analysis revealed 47 distinct profiles that were grouped in 6 groups, with 5 samples that were not assigned to any of the groups based on >80% similarity criterion. The largest group comprised 29 out of 55 isolates (52.7%). There were 3 more groups comprising 6, 5, and 5 isolates, respectively. Two more groups contained 2 isolates each, while there were 5 samples which could not be assigned to any group. The distribution of strains shows correlation with the location of hospital departments where the isolates were collected, which implies that most of the infections are the result of the spread of the epidemic strains of the bacteria within the hospital.

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## WORKSHOP – PRACTICAL PART
## *KLEBSIELLA PNEUMONIAE* GENOTYPING BY PFGE

### REAGENTS

**SE buffer**

| Working solution | Stock | 50 ml | 500 ml |
|---|---|---|---|
| 75 mM NaCl | 0.75M | 5 ml | 50 ml |
| 25 mM EDTA | 0.25M | 5 ml | 50 ml |
| $H_2O$ | | 40 ml | 400 ml |

Autoclave and store at RT

**Lysis buffer 1 (optional for Gram-negative bacteria)**

| Working solution | Stock | 10 ml | 50 ml |
|---|---|---|---|
| 6 mM Tris-HCl | 1M | 60 µl | 300 µl |
| 100 mM EDTA pH 7.5 | 0.5 M | 2 ml | 10 ml |
| 1M NaCl | 5M | 2 ml | 10 ml |
| 0.5% (w/v) Brij 58 | 5% | 1 ml | 5 ml |
| 0.2% (w/v) SDS | 10% | 200 µl | 800 µl |
| 0.5% (w/v) lauroyl sarcosine | 10% | 500 µl | 2.5 ml |
| 0.5 mg/ml lysozyme* | 100 mg/ml | 50 µl | 250 µl |
| $H_2O$ | / | to 10 ml | to 50 ml |

* lysozyme is added prior to use

**Lysis buffer 2**

| Working solution | Stock | 10 ml | 50 ml |
|---|---|---|---|
| 1% (w/v) lauryl sarcosine | 10% | 1 ml | 5 ml |
| 500 mM EDTA pH 9.5 | 1M | 5 ml | 25 ml |
| 0.5 mg/ml Proteinase K* | 20 mg/ml | 250 µl | 1.25 ml |
| $H_2O$ | / | 3.75 ml | 18.75 ml |

Filter sterilise and keep at RT.

*Proteinase K is added prior to use

**TE buffer, pH 7.5**

| Working solution | Stock | 50 ml | 500 ml |
|---|---|---|---|
| 10 mM Tris HCl | 1M Tris HCl pH 7.5 | 500 µl | 5 ml |
| 10 mM EDTA | 0.5 M EDTA pH 7.5 | 1 ml | 10 ml |
| $H_2O$ | / | 48.5 ml | 485 ml |

**10XTBE buffer**

| Reagent | Amount |
|---|---|
| Tris | 108 g |
| Boric acid | 55 g |
| 0,5M EDTA pH 8 | 40 ml |
| $H_2O$ | to 1 L |

Xba I restriction enzyme (NEB)
NEB4 buffer
BSA

## PROTOCOL

### Day 1 - Agarose plug preparation and cell lysis

- Inoculate 1 colony of *Klebsiella pneumoniae,* grown overnight on Blood agar plates, in 10 ml of BHI medium, and grow until cultures reach OD 600 of around 1.0 (0.8-1.2) (usually around 3 hours).
- Sterilise the plug moulds under UV for 15 min. (in the laminar hood)
- Prepare 2% LMP agarose in SE buffer
- Centrifuge bacterial cultures for 10 min at 1500 g, discard supernatant, and wash 2 times with SE buffer
- Prepare 2% LMP agarose in SE buffer and place in the 50-56°C water bath (or thermoblock)
- Dissolve bacterial pellet in 150 µl of SE buffer, and also place them in the 50-56°C water bath
- Mix 150 µl of bacterial suspension with equal volume of 2% LMP agarose by pipetting, and immediately transfer the mixture to the plug forming moulds
- Leave at 4°C for 10 min for the agarose to polymerase
- Incubate the plugs in lysis buffer 1 for 1 hour to overnight (optional for gram negatives) at 37°C
- Wash plugs 2 times for 15 min in 50 mM EDTA pH 9,5 at room temperature
- Incubate in lysis buffer 2 at 56°C overnight

**Day 2 - Restriction, sample loading, gel preparation and electrophoresis**

- Wash plugs 3 times for 30 min in TE buffer at 4°C (plugs can be stored in TE buffer at 4°C for up to one year)
- Cut 1 mm slices of plugs and incubate in 1Xrestriction buffer (1X NEB4, 1XBSA) for 30 min at 4°
- Transfer slices to the fresh restriction buffer and add 20 U of Xba I restriction enzyme
- Incubate for 2 hours (to overnight) at 37°C
- Cut three 1 mm slices of size standard (λ ladder, BioRad)
    - incubate 10 min at 45°C in the water bath; transfer to ice and keep on ice until loading on the comb
- Load gel slices on the comb (load standard in the lanes 1, 8, and 15)
- Prepare 100 ml of 1% agarose (Pulsed Field Certified Agarose, BioRad) in 0.5XTBE
- Pour the gel, and let it polymerase for 30 min.
- Prepare 2.2 L of 0.5XTBE electrophoresis buffer, pour into electrophoresis chamber, switch on the pump and cooling unit, and cool the buffer down to 14°C
- When the buffer is cooled down to 14°C transfer the gel to the chamber
- Set electrophoresis parameters:
    - Temperature: 14°C
    - Initial switch time: 5 s
    - Final switch time: 30 s
    - Gradient: 6V
    - Angle: 120°
    - Run time 20 h
- Run electrophoresis overnight

**Day 3 - gel staining and documentation**

- Stain the gel in 0.5 µg/ml EtBr solution in 0.5XTBE for 30 min
- Remove the buffer from the electrophoresis chamber, and wash it with sterile distilled water for 15 min
- Distain the gel 30 min in distilled water
- Photograph the gel on UV transilluminator
- Analyze the gel with GelCompar II software