Molecular methods in Environmental Microbiology

Dr Apostolos Vantarakis
Ass. Professor
Lab of Hygiene and Environmental Protection
Medical School,
Democritus University of Thrace, Greece
Identification and typification of microorganisms is important for:

a) the prevention, the diagnosis, and the treatment of contagious illnesses
b) the determination of the source of the pollution of the environment
c) the assessment of the risk for public health
d) the evaluation of the epidemiology of a disease.
Method characteristics for the fast detection, identification and typification of microorganisms

✓ Fast
✓ Sensitive
✓ Specific
✓ Reliable
✓ Effective
✓ Easy to learn
Techniques which are used in a microbiology laboratory

a) Cell culture

b) Non cell culture
   a. Immunofluorescent
   b. Radioimmunity (RIA)
   c. ELISA
   d. Flow cytometry

e. Molecular methods
   a. Dot Blot  b. Slot Blot  c. Southern Blot
   d. In situ hybridization  e. PCR and applications
   f. Real time PCR  g. Microarray
Typification methods in general...

- Phenotypic
  - Antibiogramme
  - Classification per biotype
  - Classification per serotype
  - Typification of bacteriocine
  - Typification of phages

- Genotypic:
  Typification uses genetic polymorphisms between microorganisms from the same species.

  The accidental recombination and mutations in the DNA create the genetic diversity between the same types that are collected by the different sources and regions, and different years.
Steps of culture methods

- Culture of the microorganisms in media
- Isolation
- Biochemical identification
- Detection of serotypes
Molecular approach in identification and typification of microorganisms...in general...

✧ **Detection of nucleic acids**
  - Plasmid analysis
  - Analysis of chromosomal DNA
  - Techniques of hybridization
  - Techniques of DNA proliferation and sequencing

✧ **Protein analysis**
  - Analysis of protein profile
  - Analysis of antigens
Criteria for the selection of an appropriate technique for the identification and typification of microorganisms

- Efficiency of typification
  - % of bacteria that can be typified
- Replicability
  - Replicability of the result
- Stability
  - Efficiency of the microorganisms to sustain their profile
- Discrimination efficiency (around 95%)
- Epidemiological identification and similarity
- Variability
- Ease to use
- Ease to interpret the results
- Low cost
Why molecular techniques are necessary for the detection of microorganisms…

- Every year **13.000.000** people (2.000.000 children) die from waterborne diseases
- American Society of Microbiology emphasize that many of the waterborne diseases are related to pathogens that enter in systems of water supply although these are controlled by water services
Molecular techniques in an environmental microbiology laboratory may be used for:

a) Detection, identification and typification of viruses in environmental samples such as water (sea, potable, bottled) or sewage.
b) Detection and identification of bacteria isolated from water in a very short time compared to the culture techniques.
c) Typification of bacteria isolated from the environment.
d) Detection of non culturable bacteria.
e) Epidemiologic study of microorganisms isolated by environmental samples.
Pathogenic bacteria are cultured in different media which are used for clinical strains.

There are several reasons for the use of PCR:
1. Difficulties in identification of bacteria
2. Large time required for the identification with culture techniques (more than two days)
3. The media required for the identification and confirmation of bacteria are very expensive
4. A few bacteria in the environment are viable but not culturable
PCR for the detection and identification of bacteria isolated from environmental samples, has been used with two ways:

1. Fast detection and identification of bacterial strains isolated (by cell culture) from the environment (e.g. Differentiation of strains isolated from the environment in pathogenic and non pathogenic)

2. Direct detection of pathogenic bacteria in environmental samples without previous cell culture.
PCR and viruses

A. Advantages of PCR against cell culture techniques in virus detection

- Increased sensitivity in the detection of viruses. 50% improvement in sensitivity
- Large variety of viruses detected
- Short time of analysis compared to virus culture techniques
- Low cost concerning the cultures
PCR and viruses

Disadvantages of PCR against culture techniques in the detection of viruses

- False negative (inhibition by several substances)
- False positive
- Non quantifiable
- Does not determine infectivity of viruses
Steps for the detection and identification of microorganisms in environmental samples

1. Sampling of suitable volume depending on the type and the turbidity of environmental samples
2. Filtration of the sample through the appropriate filter
3. Application on of the following steps depending on the microorganism involved in the sample
   a) Culture of bacteria of the sample or
   b) Isolation of nucleic acid of microorganism
   c) For viruses condensation of the microorganisms in smaller volume of water and then isolation of nucleic acid
4. Detection of nucleic acid of microorganism of the samples (usually with PCR)
5. Confirmation of the result, identification and typification of the microorganism detected
Molecular techniques applied in the analysis of environmental samples

**Blot hybridization assay**

Disadvantages

1. Small sensitivity related to the PCR assay
2. High not specific signal because interference from various factors of environmental samples
In situ Nucleic acid Hybridization

Advantages

1. Big sensitivity and specialty
2. Detection of contagious viruses
3. Localization of nucleic acid in the cell of host
4. Shorter than the culture
PCR in environmental samples

Advantages of PCR

Fast

Sensitive (only small quantities of nucleic acids detected)

Reliable

Specific (depending on the primers)

Non culturable microorganisms are detected
...PCR in environmental samples

Disadvantages of PCR

- False positive results (contamination especially in the case of nested PCR)
- False negative results (because of the presence of inhibitors)
- Specialized personnel and laboratories are required
- Non pathogenic microorganisms are also detected
- Non quantifiable method
1. Nested PCR

It has been used extensively

- for confirmation of the initial result of PCR
- for the identification of microorganisms
- for the rejection of false positive results of 1\textsuperscript{st} PCR (because of dilution of inhibitory substances of PCR)
- for the rejection of false positive results

In nested PCR primers are used (more specialized) which amplify a smaller part of the 1\textsuperscript{st} PCR.
2. Multiplex PCR

Several primers are used simultaneously for the detection of several microbes in one PCR

Advantages

- Economy
- Speed of analysis
- Detection of several microbes simultaneously
3. PCR-Restriction Enzyme Analysis (PCR-REA) or PCR-RFLP

- Depends on the amplification of specific part of DNA
- Followed by enzymatic digestion of PCR product by one or more restriction enzymes.
- With this way, polymorphisms are created (RFLP) which help to the identification of specific strain
ARDRA

Amplified Ribosomal DNA - Restriction Analysis

Genome

3' Primer

16 S

23 S

5' Primer

PCR-Amplification with primer pair
Digestion with restriction enzyme

Gel electrophoresis

Species-specific patterns
4. Cell Culture-PCR

- Combination of culture techniques and PCR.
- Useful for the detection of viruses in environmental samples considered negative for the detection of viruses by PCR because of inhibitory substances.
- The culture before PCR reduces the presence of the inhibitory substances.
- Determines the infectivity of viruses
5. Randomly Amplified Polymorphic DNA (RAPD)

- Random primers (usually 10mer oligos) which can hybridised in different parts of the genetic material.
- Result of this method is the development of a profile of characteristics bands depending on the strain.
- Identification and typification of the strain is made relating to the number and type of number of bands.

Advantages:
- No specific knowledge of the DNA sequence is required
- It can be applied in any microorganism,
- Fast (48 hours),
- Not very expensive,
- Easy to use.

Disadvantages:
- Reproducibility of the results
RAPD: Random Amplified Polymorphic DNA Markers

Analysis of DNA markers for strain-specific patterns.

Genome

PCR-Amplification and Gel electrophoresis

Strain-specific patterns
RAPD in strains of Pseudomonas spp.
RAPD for the discrimination of *E. coli* human or animal origin
Some of our applications of the molecular techniques in environmental samples

- Detection and typification of environmental mycobacteria in water
- Development of a method for the simultaneous detection of Salmonella spp. και Shigella spp. In mussels (multiplex PCR)
- Development of molecular methods for the detection of viruses and phages in shellfish
- Identification of Pseudomonas strains isolated from water samples with the comparison of electrophoretic patterns of proteins and their genetic profiles with RAPD
- Development of techniques for the discrimination of origin of E. coli isolated from the environment with RAPD-PCR and MAR.
- Detection, identification and typification of Enteroviruses, Adenoviruses, HAV, Rotaviruses, Noroviruses isolated from sewage. PCR και Sequencing is used.
- Detection of parasites (Cryptosporidium and Giardia) in potable water
- Development of a method for the simultaneous detection of Vibrio spp., Salmonella spp and E.coli in environmental samples (multiplex PCR).
Detection and typification of mycobacteria in water by PCR-REA

- Filtration of the water sample (200 ml)
- Pre-enrichment in Middlebrook broth
- Cultivation of mycobacteria in Middlebrook Agar
- Isolation of DNA from the colonies grown
- PCR (with hsp69 primers)
- Digestion of PCR products with restriction enzymes (BstEII, HaeIII)
- Identification of strains based on the profile according to an algorithm
**Results (I)**

**Figure 1.** PCR product (439 bp) with use of primers by the gene hsp65.

Lane 1: φX174 x HaeIII (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp), Lane 4: negative control, Lane 2, 3, 5, 6, 7 PCR products (439 bp) Lane 2: 10^2 cfu/ml, Lane 3: 10^3 cfu/ml, Lane 5: 10^4 cfu/ml, Lane 6: 10 cfu/ml Lane 7, 8, 9: PCR products after pre-enrichment of the sample, 1 cfu/ml, 10 cfu/ml and 100 cfu/ml correspondingly.
Results of environmental mycobacteria detected in water

**Upper Gel:** PCR products digested by Bst EII

**Bottom Gel:** PCR products digested by Hae III

Lane 1: Marker φX174 x HaeIII
Lane 2: M. chelonae
Lane 3: M. chelonae
Lane 4: Unidentified
Lane 5: M. gordonae
Lane 6: M. chelonae
Lane 7: M. gastrii/M. kansasii
Lane 8: M. chelonae
Lane 9: M. gordonae
Algorithm for the identification of mycobacteria
(Adapted by Telenti et al.)
Development of Multiplex PCR for the detection of Salmonella and Shigella spp.

Fig. 1 Agarose gel electrophoresis of PCR products from different template DNAs. The PCR product of *Salmonella* spp. is 275 bp and the PCR product for *Shigella* is 215 bp. The sources of the DNA in the lanes are as follows: Lane 1: φ×174× HaeIII marker; lane 2: negative control; lane 3: positive sample for *Salmonella*; lane 4: positive sample for *Shigella*; lane 5: multiplex PCR product for *Salmonella* and *Shigella*.

Fig. 2 Agarose gel showing sensitivity of multiplex PCR assay for detection of *Salmonella* spp. and *Shigella* spp. in mussels. Lane 1: marker 100 kbp ladder; lane 2: negative control; lanes 3–8: *Salmonella* and *Shigella* (10^7, 10^6, 10^5, 10^4, 10^4, 10^2 and 10 bacteria ml homogenate^-1, respectively).
SDS-electrophoresis of Ps. Aeruginosa strains isolated from sea water
SDS-electrophoresis of proteins from *Ps. Aeruginosa* strains isolated from bottled water
The most important problems caused by PCR use in the monitoring of environmental samples are:

a) it is not easy to know the true number of microorganisms in environmental samples

b) microorganisms detected may not be viable or contagious

c) inhibition of PCR by substances
For the solution of the first problem:
(it is not easy to know the true number of microorganisms in environmental samples)

1. Taqman system (Perkin Elmer, 1998)

2. Light Cycler system (Boehringer Mannheim, 1998)

These systems are using the detection of changes in the levels of fluorescence of detectors or DNA binding the fluorescent stains.
For the solution of the second problem:
(microorganisms detected may not be viable or contagious)

- RT-PCR has been used with which m-RNA of the microorganisms has limited life.
- Their detection shows recent contamination of the sample
For the solution of the third problem
(inhibition of PCR by substances)

- Columns for the purification of nucleic acid etc.
- **Nucleic Acid Capture:** The nucleic acid of the microorganism hybridized with oligo nucleotides
Basic steps in Real Time PCR

- DNA is amplified with fluorescent detector
- The instrument stimulate the stain with light
- The optical photometer reads the amount of fluorescence produced
- The intensity of fluorescence is analogous of the DNA amplification
Advantages of Real time PCR

- Reduce test and mistakes
- May determine the amount of DNA - είναι ποσοτική μέθοδος
- Negative and positive samples are determined
- Determine the purity of PCR product
- Improves the sensitivity of the PCR reaction
- No gels
TaqMan® System

✅ Uses Taq polymerase with nuclease
✅ The detector hybridized in the aim
✅ Polymerase cuts the detector
1) Denaturation

2) Hybridization

3) Extension

Taqman Probe

- Primer
- Fluorophore
- Quencher
- Polymerase

F (Fluorophore)
Q (Quencher)
Smart Cycler® System

16-Site Processing Block
Characteristics of Smart Cycler®

- Detection with real time PCR
  - Simultaneous amplification and detection
  - Reproducible, quantifiable results
  - High speed
  - Reduction of each cycle of this reaction
  - More experiments in parallel
  - Improvement of the work in the lab
  - Number of samples to be analysed 250 samples/ day
IMS method
(C. parvum & G. lamblia)

1. Capture
   Add Dynabeads anti-Cryptosporidium
   Dynal L10 tube
   Sample + SL™ buffer

2. IMS
   Pour off supernatant
   Dynabeads anti-Cryptosporidium-oocyst complex

3. Transfer & Wash
   Transfer Dynabeads crypto-oocyst complex to 1.5 ml tube
   1 ml SL™ buffer

4. IMS
   Aspirate buffer

5. Dissociation
   Add acid and vortex to dissociate complex

6. Stain & Enumeration
   Transfer dissociated oocysts to Dynal Spot-On microscope slide and stain
C. PARVUM & G. LAMBLIA
IMS method

- **Upper photo:** Traditional techniques
- **Lower photo:** Sample treated with Dynabeads GC Combo

Green: G. duodenalis
Small red/yellow: C. parvum
Big yellow yellow/red: C. muris
C. PARVUM & G. LAMBLIA
Immunofluorescence
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Genetic Fingerprinting of Microorganisms

Sample, e.g. from
- Environment
- Hospital
- Food

Grow microorganisms on solid medium

Prepare crude lysates

ARDRA = Amplified Ribosomal DNA-Restriction Analysis

Group isolates according to species

Analyses of

RAPD = Random Amplified Polymorphic DNA

Differentiate strains within species groups
Analysis of Fingerprint Patterns

Electrophoresis gel

Video Camera

analog data

Scanner

Digitizer Board

Automated DNA sequencer

analog data

Digital Fingerprint Data

computer

code

Analysis Software

Background Subtraction
Standardization
Data Compression

Fingerprint data base

Comparison of Fingerprints
Phylogenetic tree construction
II. Genomics

What is Genomics?

- An operational definition:
  - The application of high throughput automated technologies to molecular biology.

- A philosophical definition:
  - A wholistic or systems approach to the study of information flow within a cell.
Molecular Technologies

- Automated DNA sequencing
- Automated annotation of sequences
- DNA microarrays
  - gene expression (measure RNA levels)
  - single nucleotide polymorphisms (SNPs)
- Protein chips (SELDI, etc.)
- Protein-protein interactions
More Buzzwords

- Functional Genomics
- Comparative Genomics
- Pharmacogenomics
- Metabolic Reconstruction
DNA microarray

- A core technology to establish a molecular marker system that used genes in the gene clusters encoding the surface antigen of pathogenic bacteria as the specific molecular marker for efficient and reliable detection of pathogens.
DNA chip microarrays

- Put a large number (~100K) of cDNA sequences or synthetic DNA oligomers onto a glass slide (or other substrate) in known locations on a grid.
- Label an RNA sample and hybridize
- Measure amounts of RNA bound to each square in the grid
- Make comparisons
  - Cancerous vs. normal tissue
  - Treated vs. untreated
  - Time course
- Many applications in both basic and clinical research
Fig. 1. Construction of DNA microarrays for microorganisms. Not shown here are the construction of oligonucleotide arrays by immobilization of oligonucleotides within a polyacryamide gel and the microelectronic technology developed by Nanogen (Table 1). DNA microarray can also be constructed with random clones for microbes if the complete genome sequence is not available.
Fig. 2. An example of a DNA microarray experiment. This false-color image shows the induction of *nar* genes and their regulation by FNR protein under anaerobic conditions in *B. subtilis*. The *nar* genes are involved in anaerobic nitrate reduction. Genes induced under anaerobic conditions are shown in red.
GeneChip Expression Array Design

Reference sequence

… TGTGATGGTGGAATGGGGTCAGAAGGACTCCTATGTGGGTCAGCAGGGCC…

DNA probe pairs

AATGGGTCAGAAGGACTCCTATGTG
AATGGGTCAGAAACGACTCCTATGTG

Reference sequence

mRNA reference

Perfect match probe cells

Mismatch probe cells

Fluorescence Intensity Image
cDNA spotted microarrays
Scatter plot of all genes in a simple comparison of two control (A) and two treatments (B: high vs. low glucose) showing changes in expression greater than 2.2 and 3 fold.
Microarray data analysis

- Clustering and pattern detection
- Data mining and visualization
- Controls and normalization of results
- Statistical validation
- Linkage between gene expression data and gene sequence/function/metabolic pathways databases
- Discovery of common sequences in co-regulated genes
- Meta-studies using data from multiple experiments
CONCLUSIONS

- Molecular techniques are fast, sensitive and reliable for the detection of environmental samples

- Viable But Not Culturable can be detected

- More than one microorganisms may be detected at the same time

- With the combination with culture techniques pathogenic and viable microorganisms may be detected