MOLECULAR TECHNIQUES FOR DETECTION OF PATHOGENS AND VIRULENCE GENES

MOLECULAR TECHNIQUES:ADVANTAGES

1- RAPID RESULTS

2- HIGH SENSITIVITY

3- VIABLE MICROORGANISM IS NOT NEEDED

4- USEFUL FOR ORGANISMS NON-DETECTED BY CONVENTIONAL METHODOLOGY

5- RESULTS ARE NOT AFFECTED BY PHENOTYPIC EXPRESSION

6- SPECIFICITY

MOLECULAR TECHNIQUES: DISADVANTAGES

1- NUCLEIC ACID DETECTION DO NOT ALWAYS INDICATE INFECTIOUS DISEASE

2- PERCENTAGE OF FALSE POSITIVE/NEGATIVE RESULTS IN SOME TECHNIQUES (PCR)

3- BEST RESULTS WITH PREVIOUS SEQUENCED GENOMES

4- NEED OF WELL-TRAINED TECHNICIANS TO AVOID FALSE-POSITIVE AND -NEGATIVE RESULTS.

5- CLINICAL INTERPRETATION

MOLECULAR TECHNIQUES

1-PLASMID ANALYSIS: detection of virulence plasmids and epidemiological studies.

2- HYBRIDIZATION WITH DNA PROBES: detection of specific genes with a high level of sensitivity.

3- DNA ARRAYS: hybridization with hundreds of probes on a microchip

4- PULSED FIELD GEL ELECTROPHORESIS: golden standard for DNA-fingerprinting.

5- POLYMERASE CHAIN REACTION: rapid and specific detection even if very few copies of target DNA/RNA are present in the sample.

6- SEQUENCING: definitive results .

7- MULTILOCUS SEQUENCE TYPING: identification of allele variations

1. HYBRIDIZATION WITH DNA PROBES

HYBRIDIZATION WITH DNA PROBES

BASIC PRINCIPLES:

1- DESNATURALIZATION OF TARGET DNA AND FIXATION ON A MEMBRANE, MICROTITTER PLATE….

2- LABELING OF THE PROBE (ssDNA)

3- HIBRIDYZATION

4- DETECTION OF HYBRIDS

HYBRIDIZATION FORMATS

1- LIQUID: tubes, microtitter plates

2- SOLID: nylon membranes
   - DOT-BLOT & SLOT-BLOT
   - SOUTHERN BLOT
   - NORTHERN BLOT

3- IN SITU: microscope slides

TYPES OF PROBES

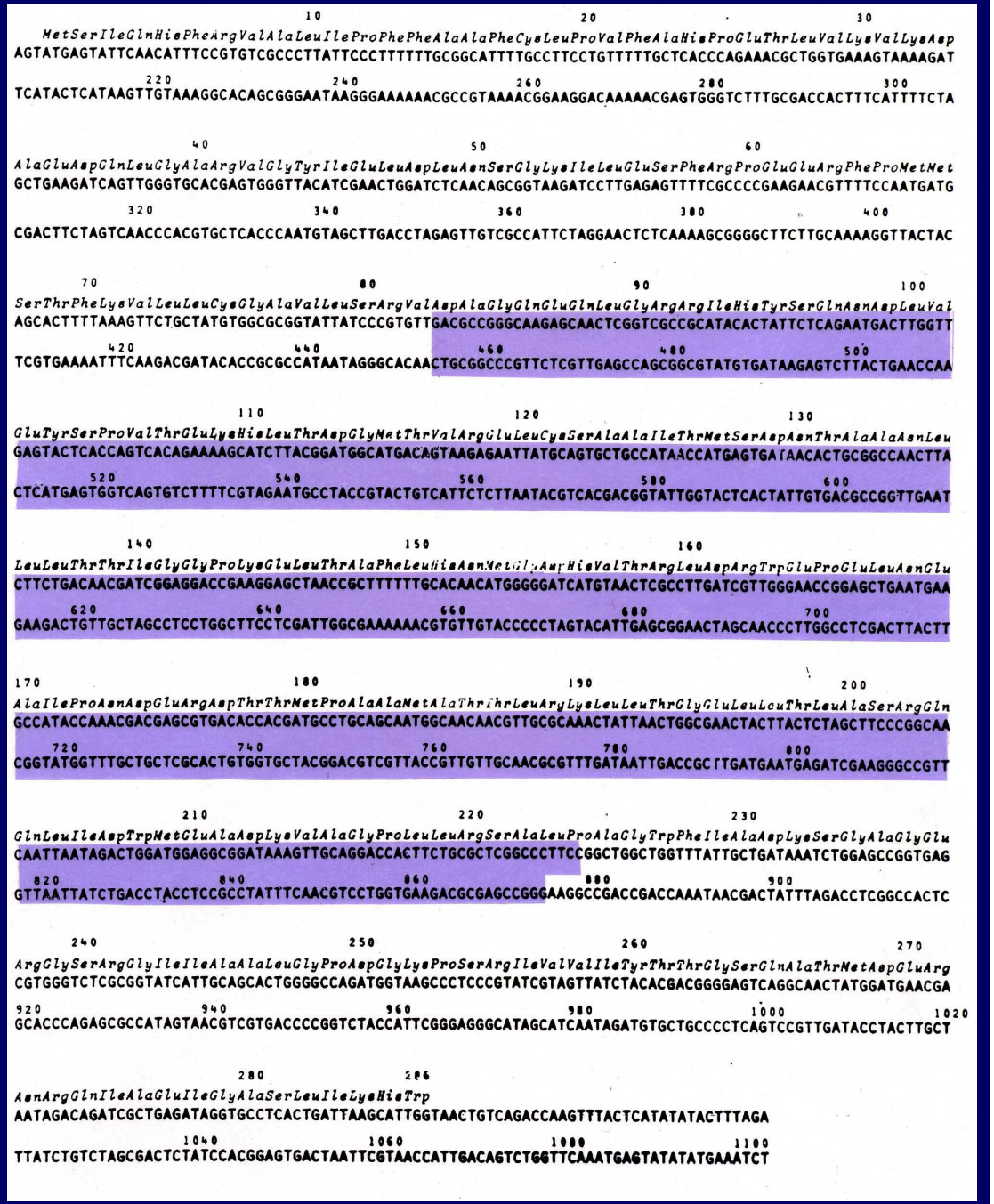
- NUCLEIC ACID:
  - DNA
  - RNA
  - NUCLEOTIDES

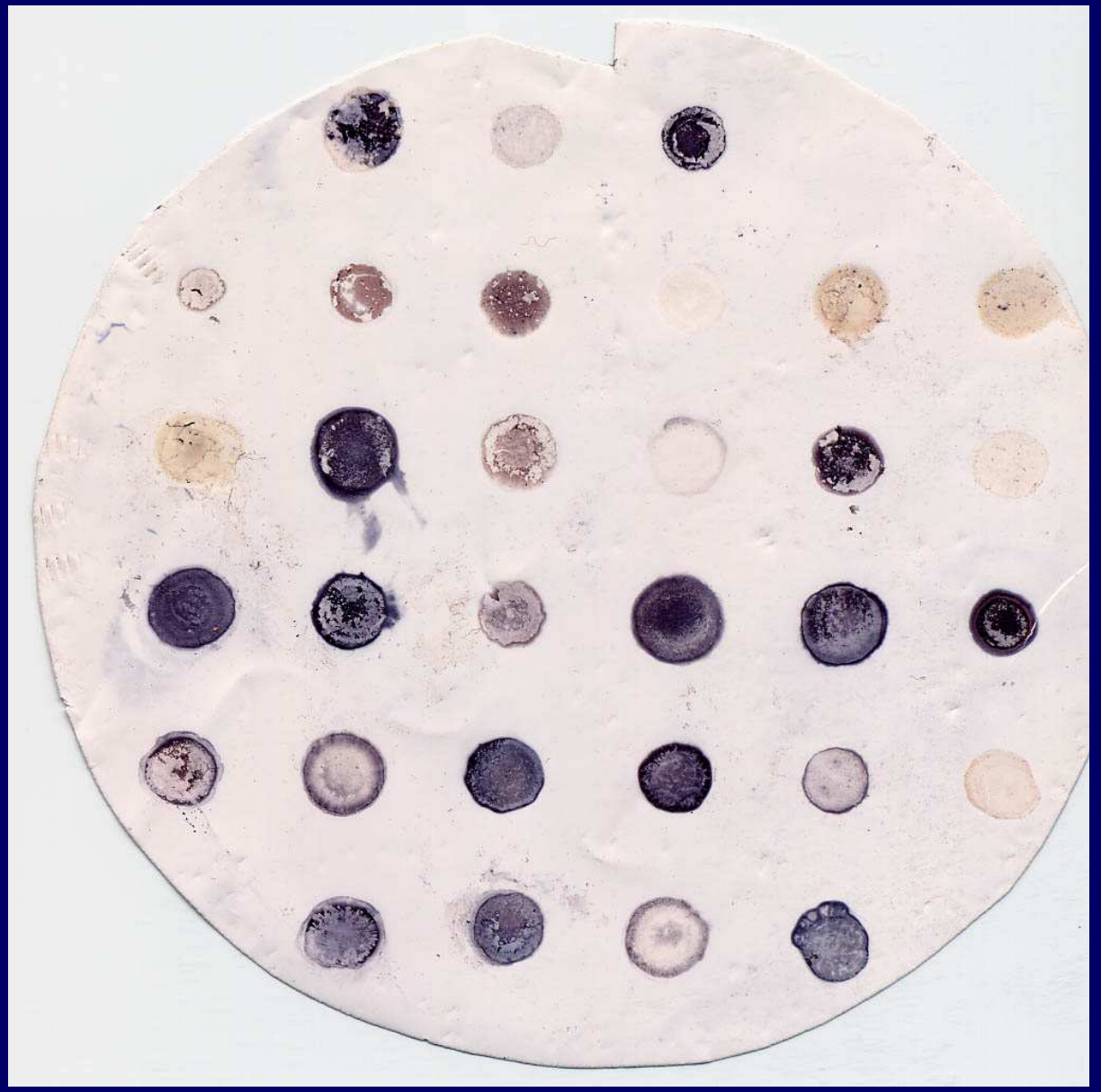
- LABELING COMPOUND:
  - DIGOXIGENIN
  - BIOTIN
  - FLUORESCENT LABELS
  - PEROXIDASE
  - ALKALIN FOSFATASE

- PREPARED BY:
  - ENDONUCLEASE DIGESTION
  - CLONING
  - PCR
  - CHEMICAL SYNTHESIS

PLASMID pBR322

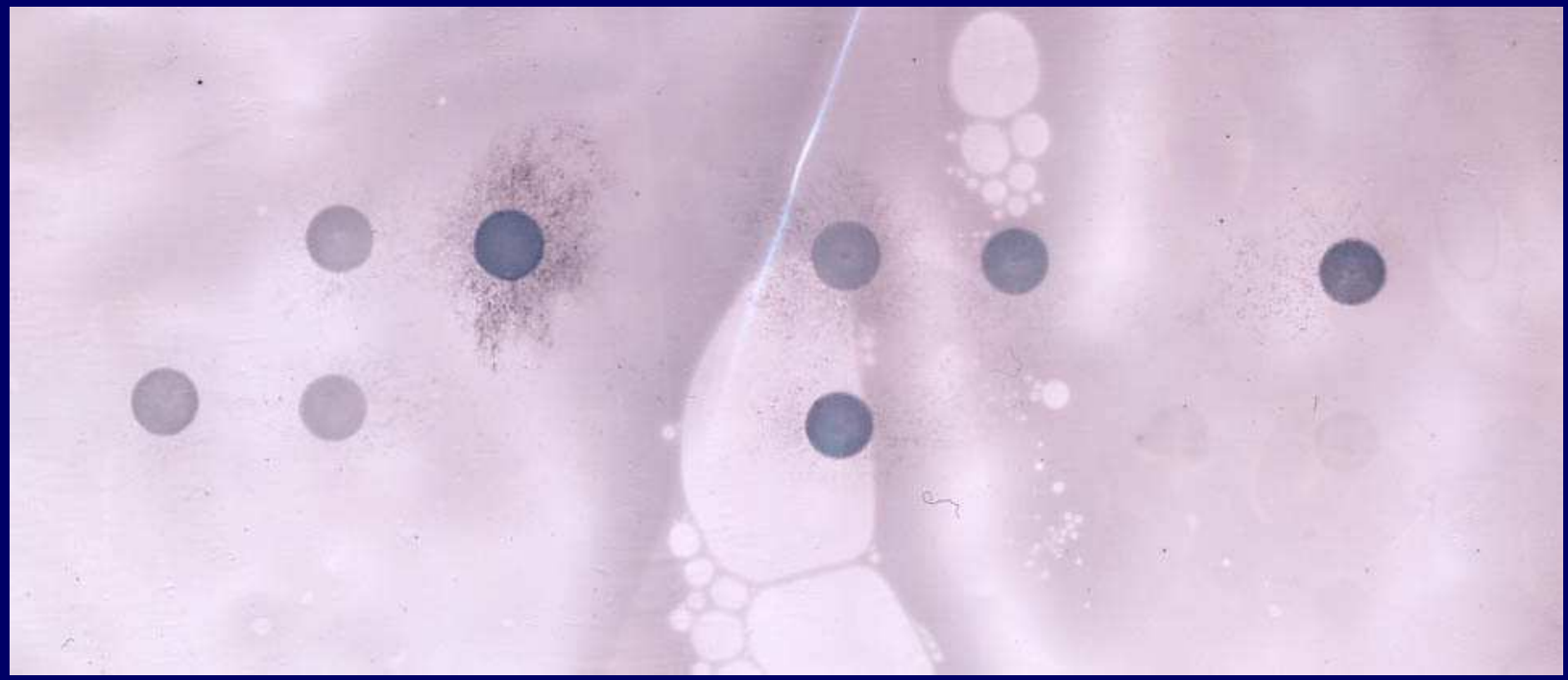
(HIGHLIGHTED THE SEQUENCE USED TO OBTAIN A TEM-TYPE β-LACTAMASES DNA PROBE)





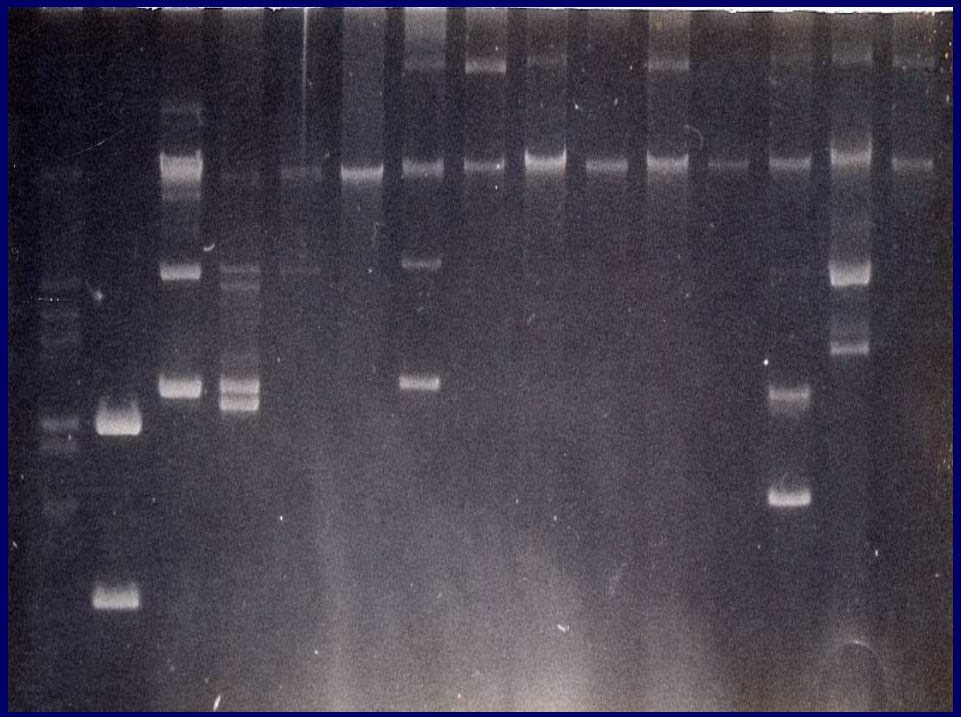
COLONY HYBRIDIZATION WITH A DIGOXIGENIN-LABELED TEM-TYPE β-LACTAMASES PROBE

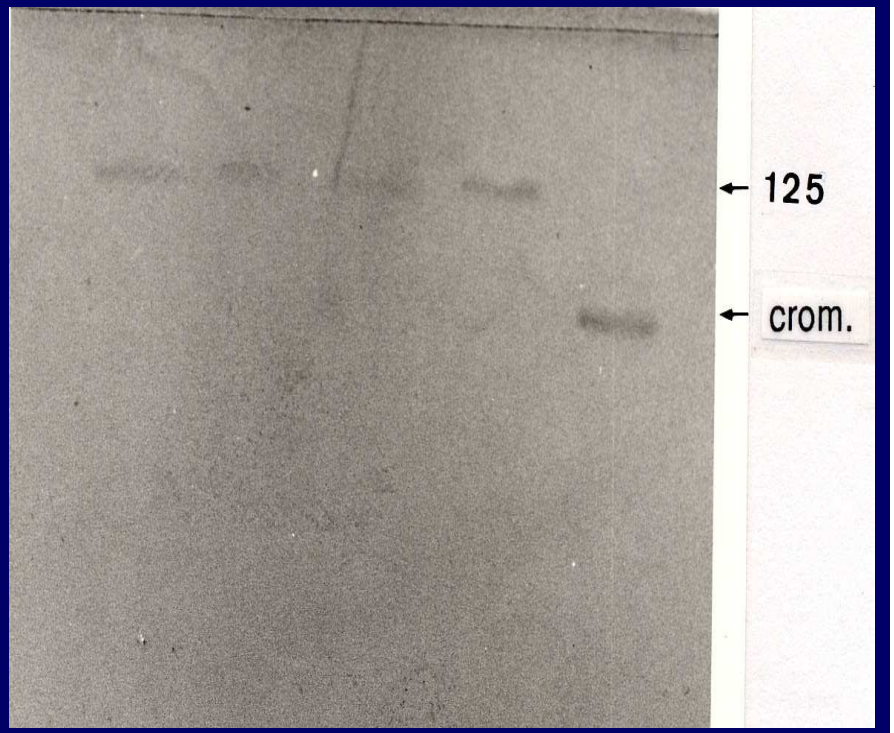
(Target DNA: crude colony lysates of *Salmonella* spp. isolates)



DOT-BLOT HYBRIDIZATION WITH A TEM-TYPE β-LACTAMASES PROBE

(Target DNA: total DNA from *Salmonella* spp. isolates)





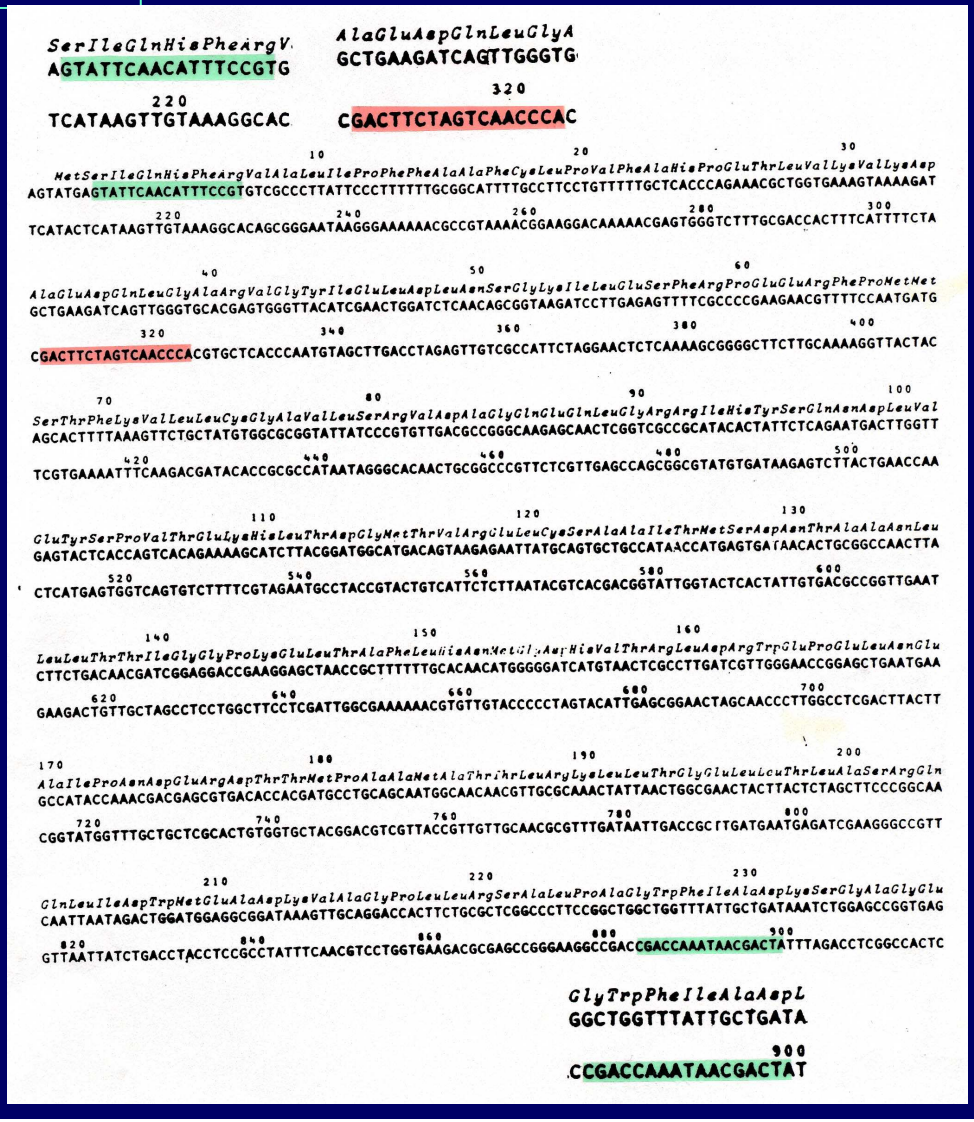
SOUTHERN-BLOT HYBRIDIZATION WITH A TEM-TYPE β-LACTAMASES PROBE

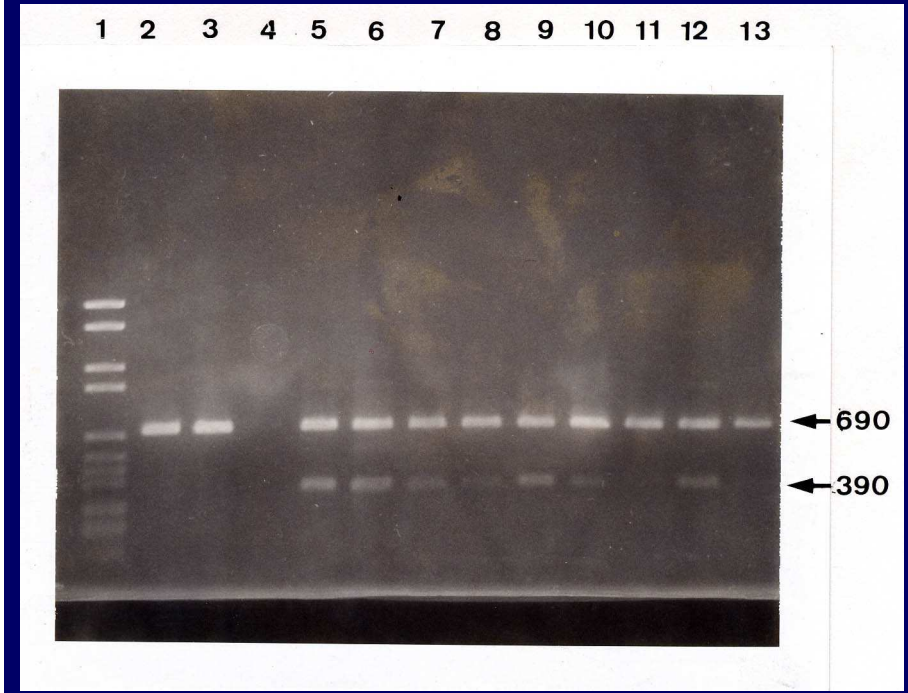
(Target DNA: plasmid DNA from *Salmonella* spp. isolates)

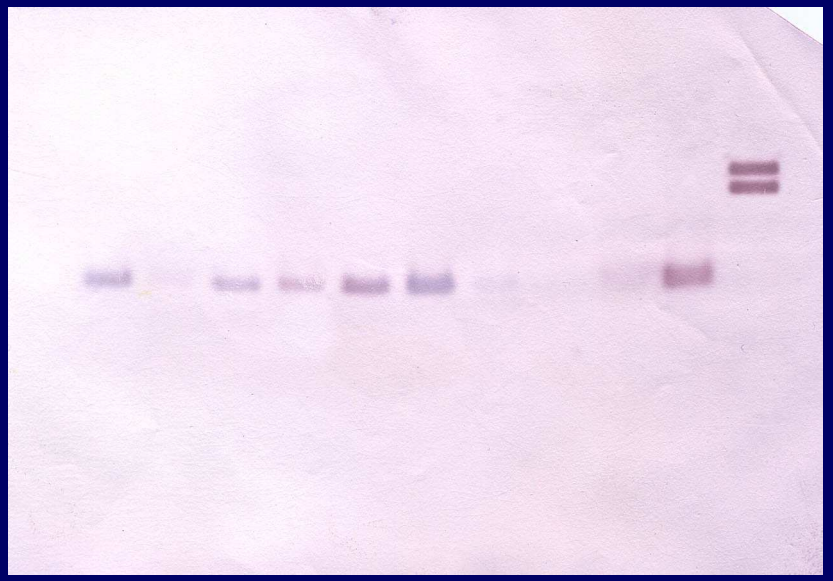
TEM-1 PROBE (Gln 37) 5'-ACCCAACTGATCTTCAG-3'

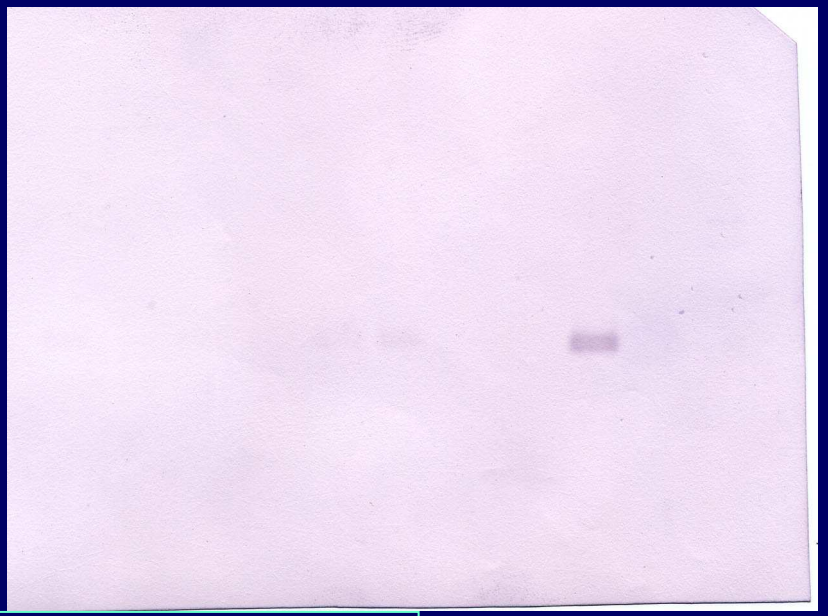
TEM-2 PROBE (Lys 37) 5'-ACCCAACTTATCTTCAG-3'

OLIGONUCLEOTIDE PROBES TO DETECT POINT MUTATIONS IN THE SEQUENCES OF THE TEM β-LACTAMASES VARIANTS



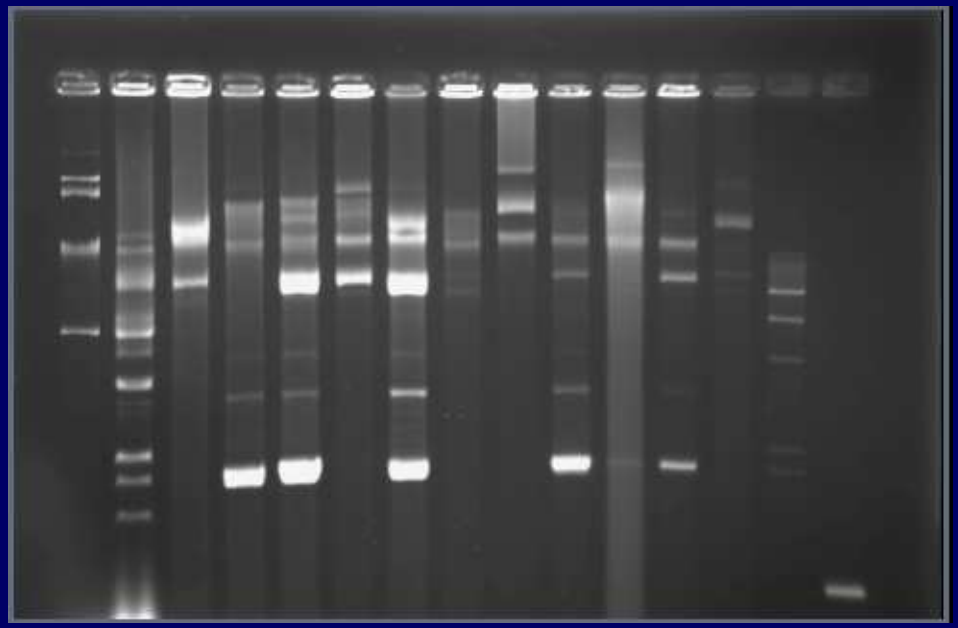


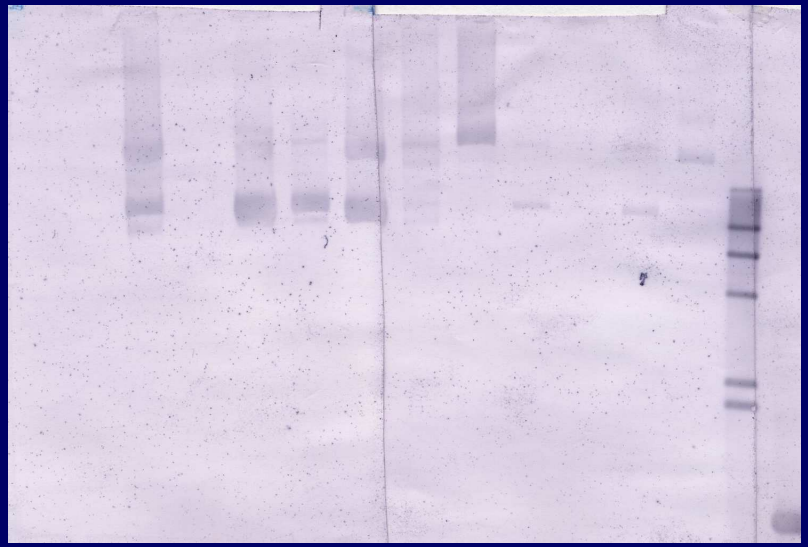




SOUTHERN-BLOT HYBRIDIZATION WITH TEM-1 & TEM-2 PROBES

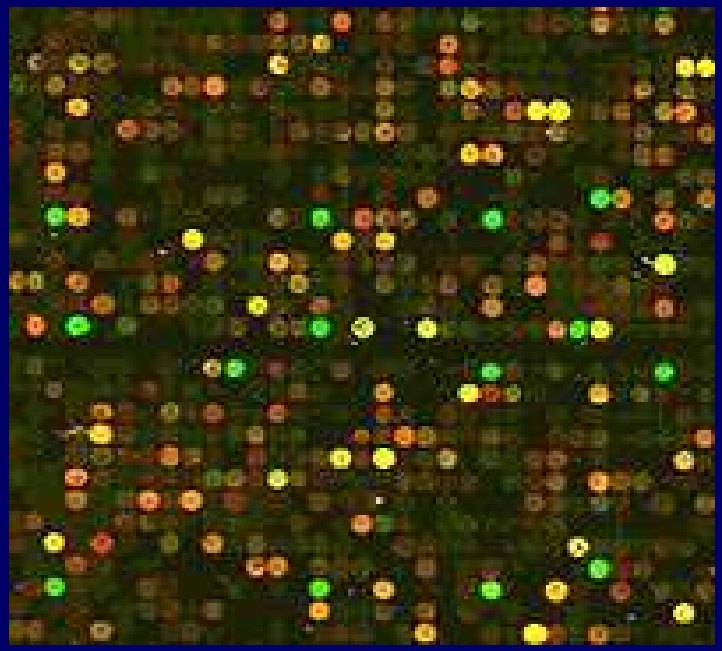
(Target DNA: amplified DNA from *Salmonella* spp. isolates)





SOUTHERN-BLOT HYBRIDIZATION USING AN OXA-40 CARBAPENEMASE PROBE WITH PLASMID DNA FROM CLINICAL ISOLATES OF *A. baumannii*

2. DNA MICROARRAYS



DNA MICROARRAYS/BIOCHIP/DNA CHIP/GENE ARRAY

-USE: ANALYSIS OF THE COMPLETE GENOME OF A MICROORGANISM

-METHODOLOGIC BASIS: HYBRIDIZATION TECHNIQUE

-FORMAT: MICROTITTER PLATES, NYLON MEMBRANES

-MANUFACTURE: IN THE LABORATORY OR USING COMMERCIAL ROBOTICS

MACROARRAY: SIGNALS > 300 MICRAS

MICROARRAY: WELLS < 200 MICRAS

APLICATIONS

1. IDENTIFICATION OF SEQUENCES (GENES, MUTATIONS..)
2. DETERMINATION OF THE LEVEL OF GENETIC EXPRESSION
3. DISCOVERY OF NEW GENES
4. DIAGNOSIS OF DISEASES
5. PHARMACOGENOMICS: DEVELOPMENT OF NEW DRUGS
6. TOXIGENOMICS: TOXICOLOGIC RESEARCH

DESIGN

1. PROBE: cDNA (500-500 bp),oligonucleotide (20-80 mer)
2. CHIP MANUFACTURE
3. LABELLED TARGET DNA (RNA, DNA)
4. EXPERIMENT: Hybridation, Fluorometry, Real-Time PCR….)
5. RESULT
6. COMPUTER-ASSISTED ANALISYS

DNA- fingerprinting

1.- PLASMID ANALYSIS (see Chapters 2 Basic Level & Chapter 8 Advanced Level .

2.- RIBOTYPING: hybridization with rDNA probes.

3.- PCR-FINGERPRINTING: rapid screening but do not allow interlaboratories comparation of the results.

4.- PFGE (PULSED FIELD GEL ELECTROPHORESIS): international golden standard.

5.- MULTILOCUS SEQUENCE TYPING: useful to identify allele variations.

DNA-fingerprinting: uses

1. IDENTIFICATION OF THE ORIGIN AND EXTENSION OF AN OUTBREAK

2. STABLISHMENT OF CROSS INFECTION AMONG PATIENTS

3. MONITORIZATION OF THE EVOLUTION OF THE INFECTION ALONG TIME (PERSISTENCE OF THE SAME BACTERIA, EMERGENCE OF NEW ONES WITH INCREASED VIRULENCE)

4. MONITORIZATION OF THE ANTIBIOTIC TREATMENTS, RESISTANCE LEVELS AND PATIENTS´S IMMUNE RESPONSE

3. PULSED FIELD GEL ELECTROPHORESIS (PFGE)

METHOD OF CHOICE FOR STUDYNG THE MOLECULAR EPIDEMIOLOGY OF BACTERIAL PATHOGENS

-DESCRIPTION: HIGH RESOLUTION TYPING TECHNIQUE DERIVED FROM THE COMBINATION OF TWO TECHNIQUES:

- -CHROMOSOMAL RESTRICTION FRAGMENT PATTERN ANALYSIS USING LOW-FREQUENCY CUTTING ENZYMES
- -PFGE: A MODIFICATION OF AGAROSE GEL ELECTROPHORESIS INITIALLY INTRODUCED FOR PROVIDING ELECTROPHORETIC KARYOTYPES OF EUKARYOTIC MICROORGANISMS

-RANGE : PFGE CAN SEPARATE DNA MOLECULES AS LARGE AS 12 Mb.

PRINCIPLES OF PFGE

1. IN RESPONSE TO CHANGES IN THE ORIENTATION OF THE ELECTRIC FIELD, LARGE DNA MOLECULES MIGRATE THROUGH THE AGAROSE MATRIX IN A ZIG-ZAG MOTION
2. DNA MOLECULES STRETCH OUT LINEARLY IN THE DIRECTION OF THE ELECTRIC FIELD
3. WHEN THE FIELD CHANGES DIRECTION, THE DNA MOLECULES INITIALLY ADOPT A PARTIAL RELAXED CONFORMATION, AND THEN FORM MULTIPLE KINKS IN THE DIRECTION OF THE NEW FIELD UNTIL THE PREDOMINANT KINK UNFOLDS AND THE DNA MOLECULE ELONGATES AGAIN TO MIGRATE IN THE NEW DIRECTION
4. THE LARGER THE MOLECULE, THE LONGER THE TIME REQUIRED FOR RELAXATION AND REORIENTATION OF ITS LEADING TIME
5. THE INTERVAL BETWEEN CHANGES OF FIELD DIRECTION (PULSED TIME) WILL PRIMARILY DETERMINE THE SIZE WINDOW FOR SEPARATION OF DNA MOLECULES BY PFGE

EQUIPMENT


BIO-RAD
Cooling Module
BIO-RAD
CHEF-DR® III System
TUBE TO CHILLER
BUFFER DRAIN TUBE

METHODOLOGY

1.- DNA PREPARATION

2.- PFGE SEPARATION (RUNNING PARAMETERS) DEPENDS ON:

- ELECTRIC FIELD
- PULSE TIME
- REORIENTATION ANGLE
- BUFFER
- AGAROSE TYPE & CONCENTRATION
- CHAMBER TEMPERATURE
- STANDARDS
- AMOUNT OF DNA IN THE SAMPLES

APPLICATIONS

1.- IDENTIFYING RESTRICTION FRAGMENT LENGHT POLYMORPHISMS

(USING LOW-FREQUENCY CUTTING ENZYMES, TYPICALLY WITH LESS THAN 30 CLEAVAGE SITES PER GENOME)

2.- CONSTRUCTION OF PHYSICAL MAPS

3.-DETERMINING THE NUMBER AND SIZE OF CHROMOSOMES (ELECTROPHORETIC KARYOTYPE)

4.- STUDY OF HIGH MOLECULAR WEIGHT PLASMIDS

5.- OTHERS: CLONING LARGE DNA USING ARTIFICIAL CHROMOSOMES; DETECTING "IN VIVO" CHROMOSOME BREAKAGE AND DEGRADATION

ANALYSIS AND INTERPRETATION OF DATA

RESTRICTION PATTERNS:

RELATED ISOLATES: SAME PATTERNS

NON RELATED ISOLATES: DIFFERENT PATTERNS

MINOR PATTERNS DIFFERENCES ARISE FROM:

* POINT MUTATIONS
* INSERTIONS
* DELECTIONS

CATEGORIES

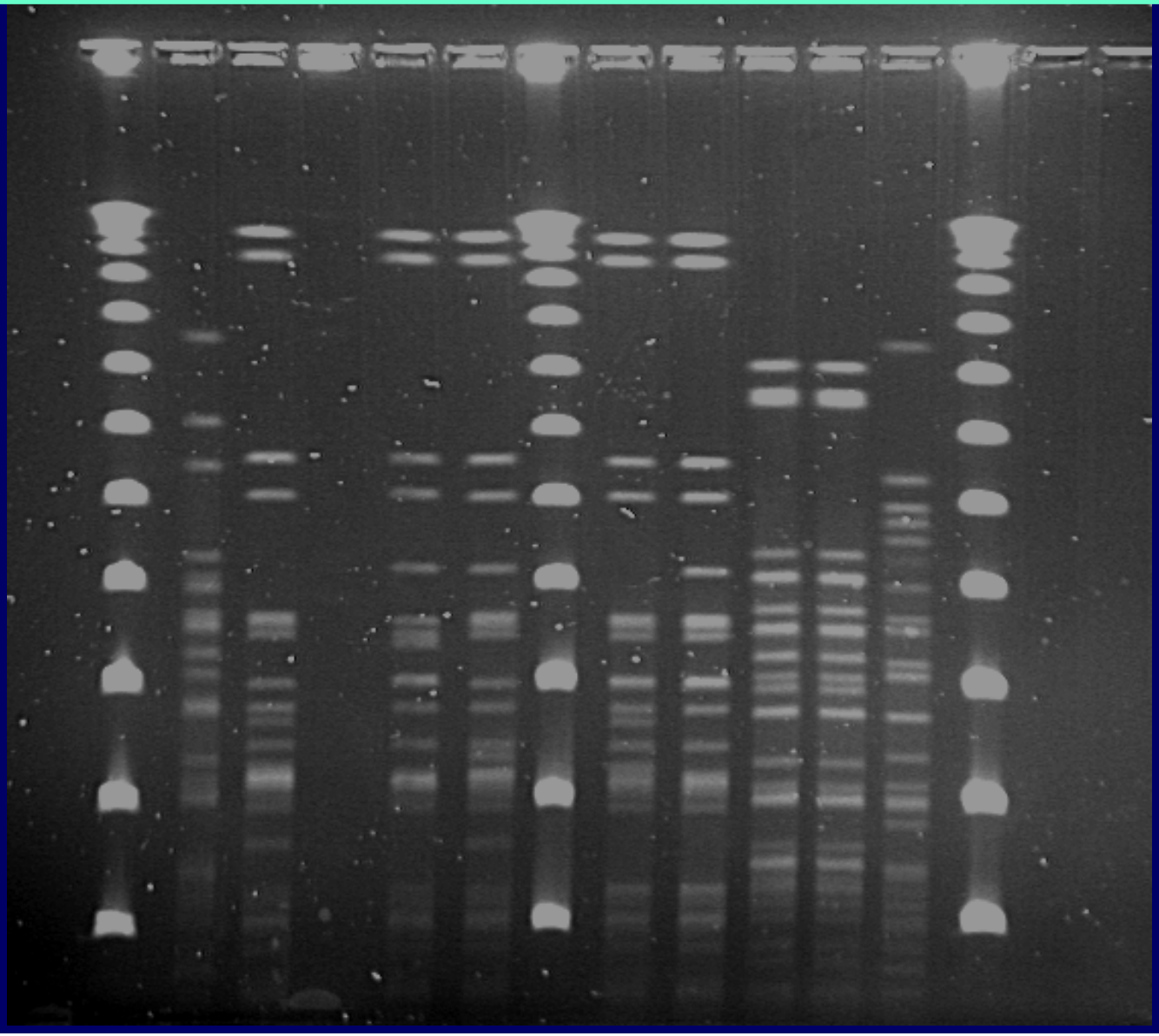
1. INDISTINGUISHABLE: isolate is part of the outbreak.

2. CLOSELY RELATED: 1 genetic difference (2-3 fragment differences)

3. POSSIBLY RELATED: 2 genetic differences (4-6 fragment differences)

4. DIFFERENT: >3 genetic differences (>7 fragment differences).

PFGE of *Apa* I digested DNA obtained from different clinical isolates of *A. baumannii*



Computer-assisted data analysis

4. MULTILOCUS SEQUENCE TYPING

MULTILOCUS SEQUENCE TYPING

DESCRIPTION: TYPING METHOD THAT ANALYSES VARIATIONS IN "HOUSEKEEPING" GENES

-DATA BASE AVAILABLE VIA WEB SITES

-RECOMMENDED FOR VALIDATION OF RESULTS OBTAINED WITH OTHER TECHNIQUES (PFGE)

-MLVA: REPETITIVE SEQUENCES THAT ARE DISTINCT IN EACH ISOLATE

5. POLYMERASE CHAIN REACTION

POLYMERASE CHAIN REACTION

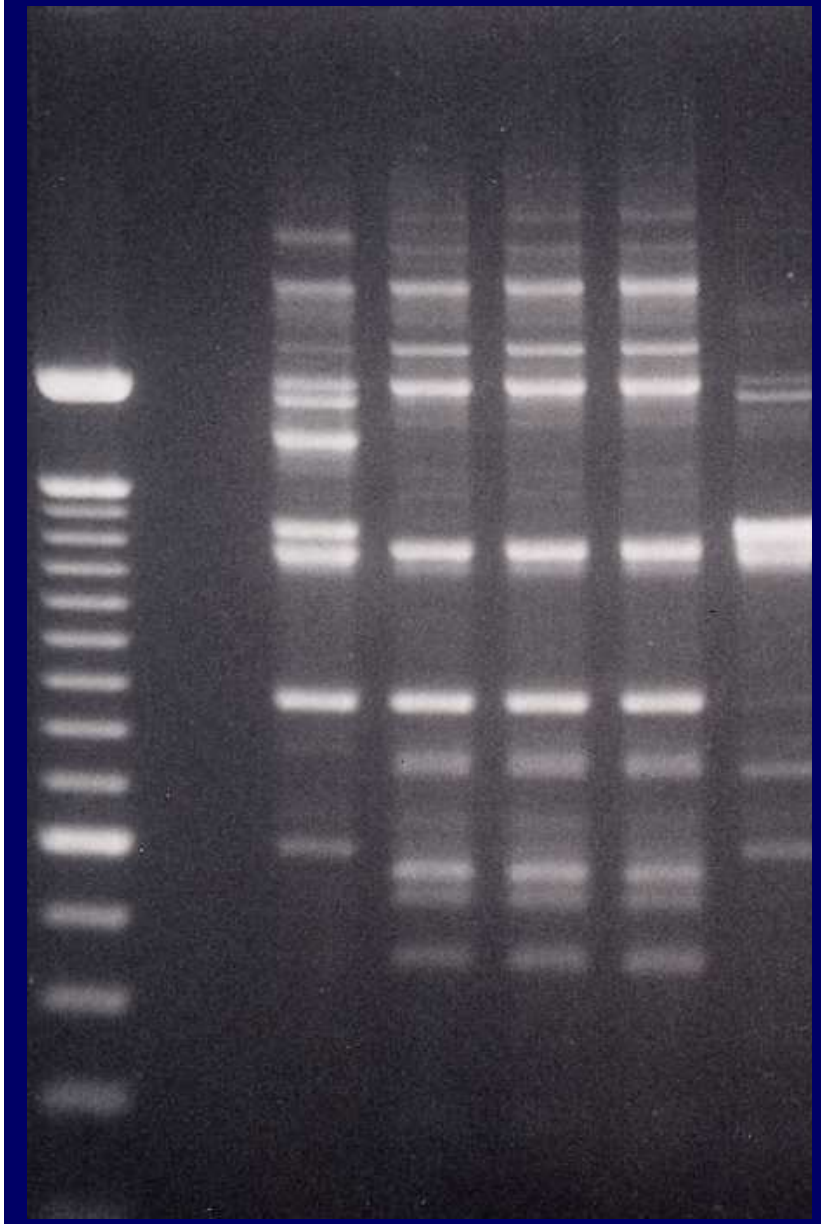
ADVANTAGES

- SENSITIVITY
- RAPID
- DETECTION OF FASTIDIOUS ORGANISMS
- NO NEED OF VIABLE CELLS
- DETECTION OF UNKNOWN SEQUENCES

DISADVANTAGES

- FALSE-POSITIVE/NEGATIVE RESULTS
- REPRODUCIBILITY
- INTERLABORATORY VALIDATION
- CLINICAL INTERPRETATION

PCR-fingerprinting

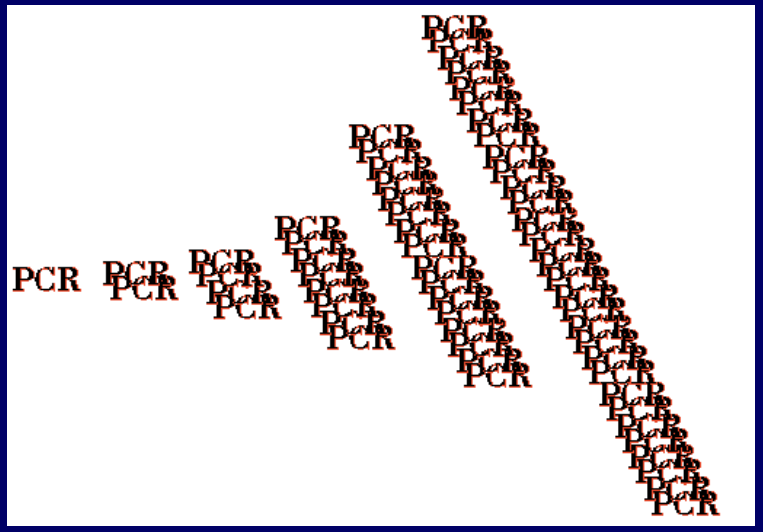


TYPING OF MICROORGANISMS USING DIFFERENT PRIMERS:

- -ERIC-PCR
- -AP-PCR

← *A. baumannii* profiles using M13 primer

MULTIPLEX-

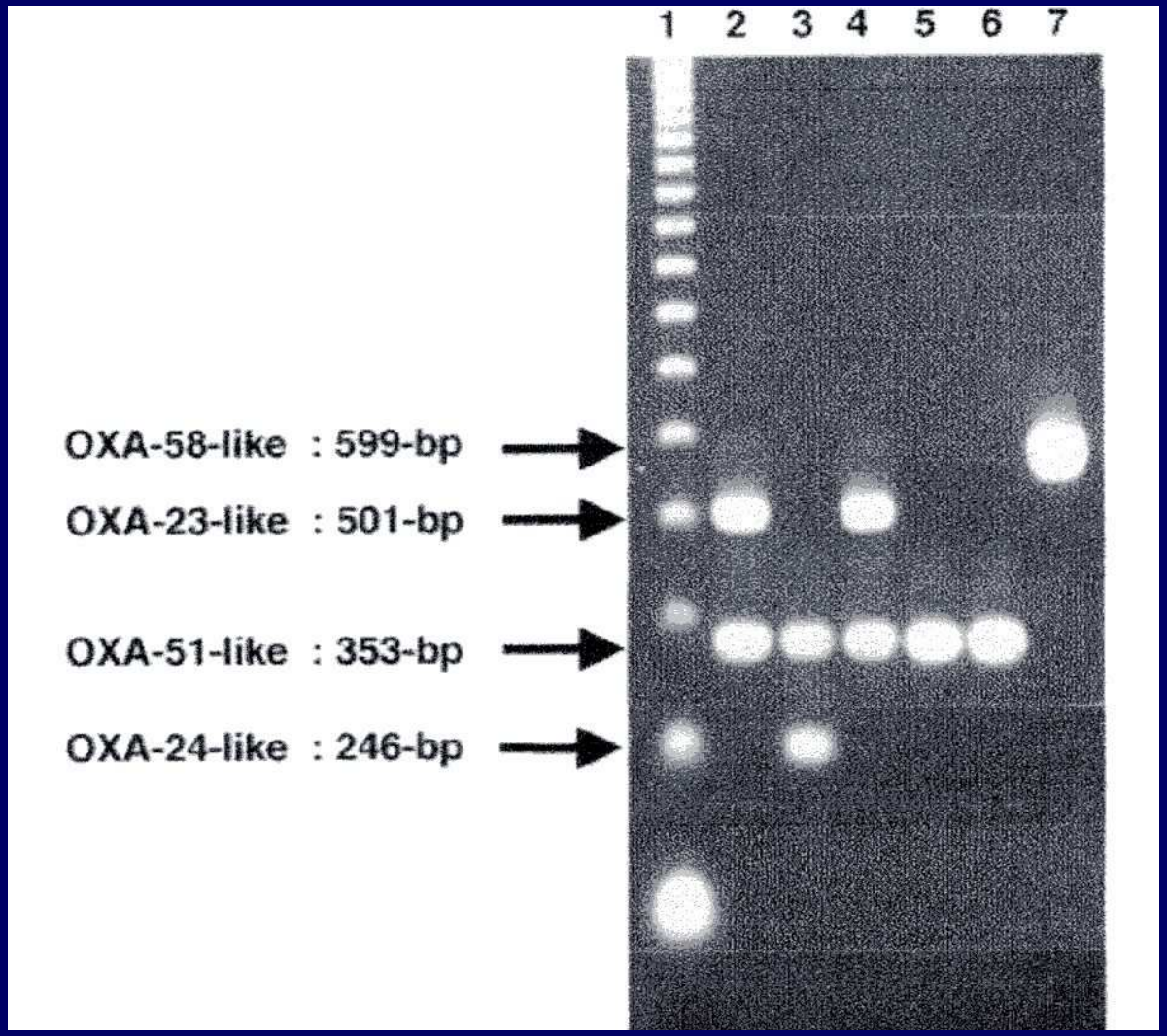


-USE OF MORE THAN TWO PRIMERS IN THE SAME EXPERIMENT

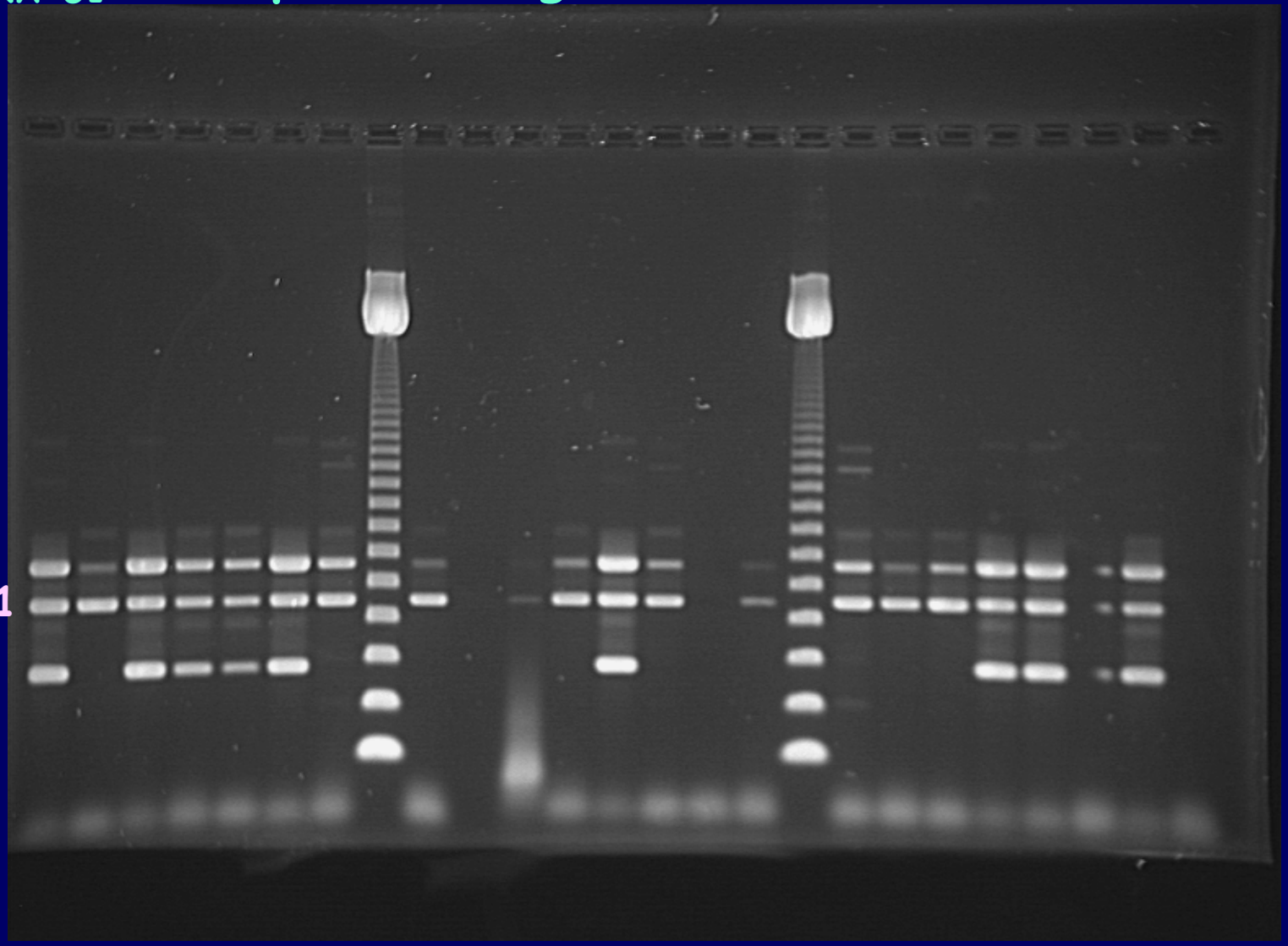
-ADVANTAGE: DETECTION OF SEVERAL GENES IN THE SAME REACTION

-DIFFICULTIES: SELECTION OF PRIMERS AND CYCLING CONDITIONS

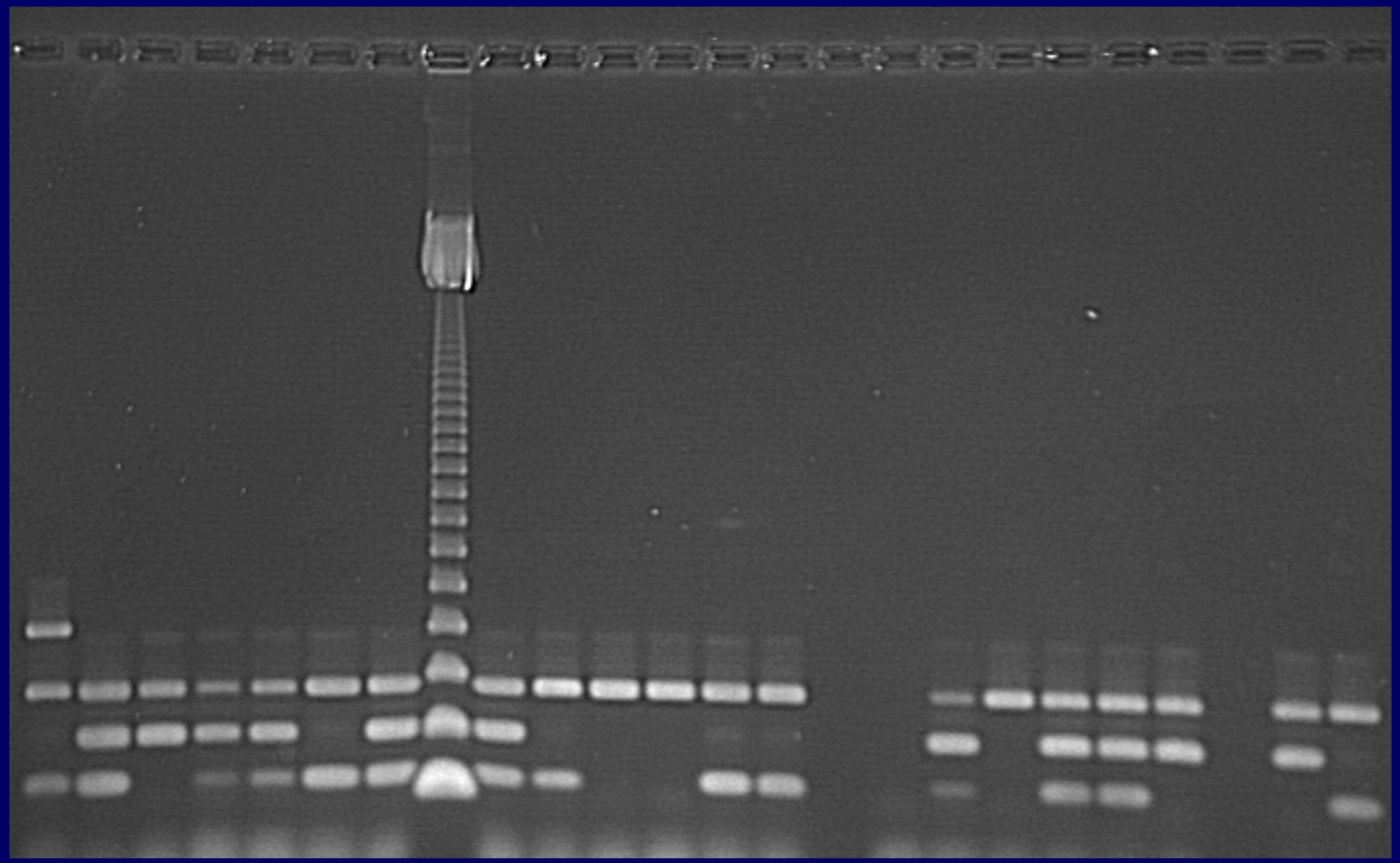
Multiplex-PCR for detection of *bla* $_{OXA-23}$, *bla* $_{OXA-24/40}$, *bla* $_{OXA-51}$ & *bla* $_{OXA-58}$ carbapenemase genes in clinical isolates of *A. baumannii*



Multiplex-PCR for detection of *csuE* & *ompA* virulence genes and *bla* $_{OXA-51}$ carbapenemase gene in *A. baumannii* isolates



Multiplex-PCR for detection of *int* 1 integrase gene and *bla* $_{OXA-23}$ *bla* $_{OXA-24/40}$ *bla* $_{OXA-51}$ carbapenemase genes in clinical isolates of *A. baumannii*



REAL-TIME PCR

1- METHODOLOGY: COMBINES PCR CHEMISTRY WITH FLUORESCENT PROBE DETECTION OF AMPLIFIED PRODUCT IN THE SAME REACTION VESSEL

2- ADVANTAGES:

- COMPLETED IN AN 1 HOUR OR LESS
- EQUIVALENT SENSITIVITY AND SPECIFICITY AS CONVENTIONAL PCR COMBINED WITH SOUTHERN BLOT ANALYSIS
- RISK FOR CONTAMINATION IS NEGLIGENT COMPARED WITH CONVENTIONAL PCR METHODS
- REQUIRES CONSIDERABLY LESS HANDS-ON TIME AND TESTING IS MUCH SIMPLER TO PERFORM

EXCELLENT SENSITIVITY AND SPECIFICITY + LOW CONTAMINATION RISK + EASY OF PERFORMANCE + SPEED

PROBE TECHNOLOGIES

1- SYBR Green: DETECTS THE ACCUMULATION OF ANY dsDNA PRODUCT

- SENSITIVITY BUT NON SPECIFIC

2- FLUORESCENT PROBES:

- SENSITIVITY & SPECIFICITY

- TYPES:
  - 5' NUCLEASE (TaqMan)
  - MOLECULAR BEACONS
  - FRET HYBRIDIZATION PROBES

- ALL RELY ON THE TRANSFER OF LIGHT ENERGY BETWEEN TWO ADJACENT DYE MOLECULES (Fluorescence resonance energy transfer)

REAL-TIME PCR: CRITICAL STEPS

1- DNA EXTRACTION: type of microorganism, presence of inhibitors, nucleases, contaminants…

2- TARGET NUCLEIC ACID SELECTION: better results when unique conserved sequences are used.

3- PRIMERS & PROBES: primers that only amplify one product will provide the best assay sensitivity.

4- ASSAY OPTIMIZATION: reagents concentration, type of polymerase, cycling conditions….

REAL-TIME PCR: QUALITY CONTROL

1- VERIFICATION AND VALIDATION

2- POSITIVE AND NEGATIVE CONTROLS

3- INTERNAL AND INHIBITION CONTROLS

4- REAGENTS

5- CONTAMINATION

QUANTIFICATION CONSIDERATIONS

1. USE OF CONTROLS IS MANDATORY:
   - EXTERNAL CONTROL: TO ESTIMATE THE AMOUNT OF TARGET IN THE SAMPLE
   - INTERNAL CONTROL: TO AVOID FALSE NEGATIVE/POSITIVE RESULTS

2. ANALYSIS OF RESULTS NEEDS COMBINATION OF ESPECTROPHOTOMETRY AND ESTATISTICAL STUDY

3. RESULTS ARE EXPRESSED IN RELATIONSHIP WITH SAMPLE VOLUME, NUMBER OF CELLS….

REAL-TIME PCR: TYPING

1- DETECTION OF: MUTATIONS, INSERTIONS AND DELECTIONS

2- METHODS:

- -SYBR Green
- -HyProbe

3- OLIGONUCLEOTIDES: BEACONS GIVE BETTER RESULTS THAN LINEAR OLIGONUCLEOTIDES

4- *DESTABILIZING MISMATCHES

C:C, C:A, C:T

*LESS DESTABILIZING MISMATCHES

G:T, G:A, G:G

REAL-TIME PCR: APPLICATIONS

VIROLOGY:

- genotyping
- asses the relationship between the viral load of a viral target and the prediction of the progression of infection to clinical disease.

BACTERIOLOGY:

- detection of fastidious bacteria
- slow-growing or poorly culturable bacteria.

MICOLOGY AND PARASITOLOGY: up to now there are not many descripcions of applications to these areas.

6. DNA SEQUENCING

MOLECULAR TECHNIQUES

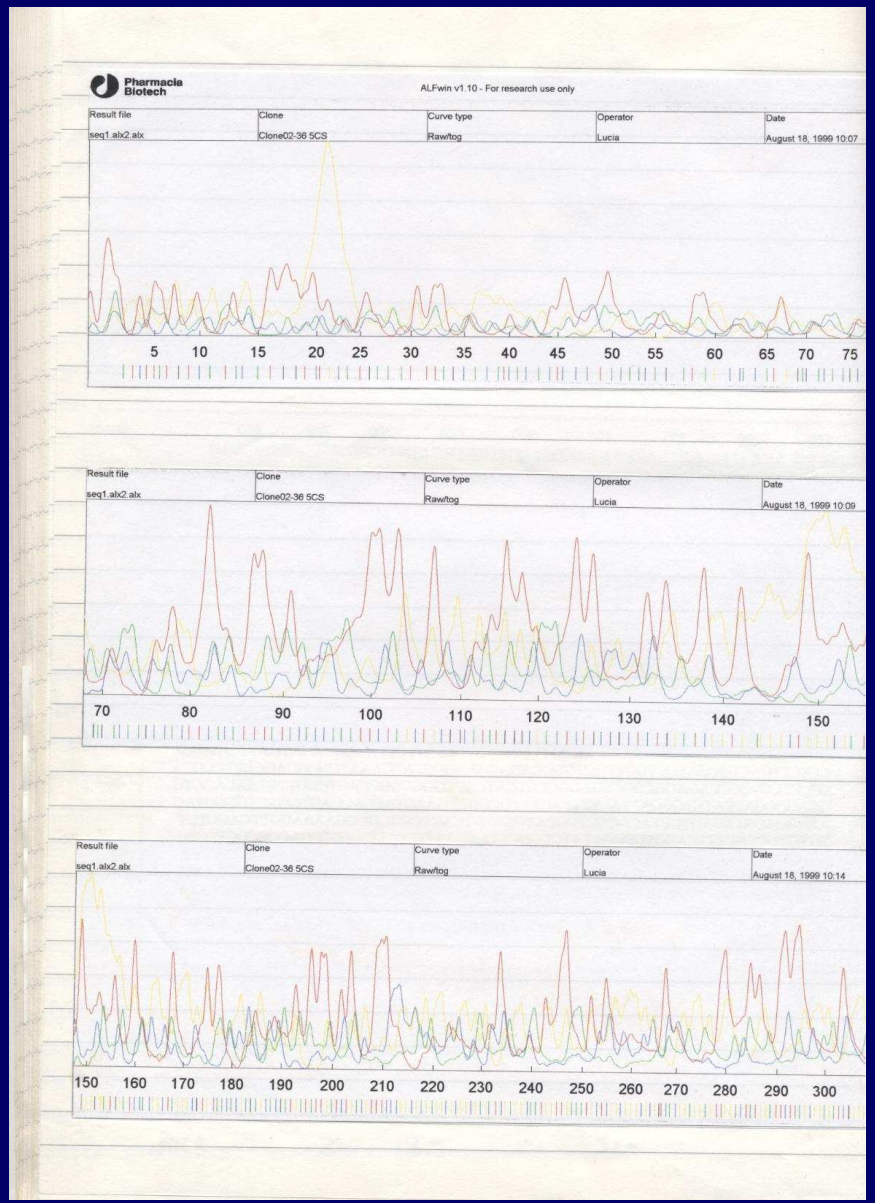
SEQUENCING:

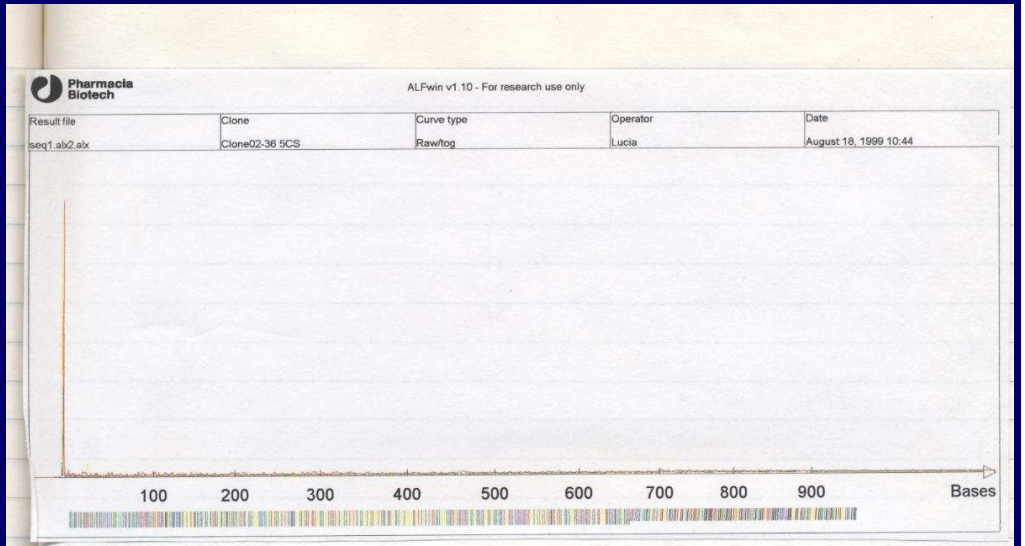
- OBTENTION OF COMPLETE NUCLEOTIDE SEQUENCES
- DEFINITIVE RESULTS
- BASIC TO DESIGN PRIMERS, PROBES……

MULTILOCUS SEQUENCE TYPING: PARTIAL SEQUENCING OF SPECIFIC ALLELE VARIATIONS

CONVENTIONAL SEQUENCING EXPERIMENT

SEQUENCE OF A CLASS 1 INTEGRON DETECTED IN ISOLATES OF *Acinetobacter baumannii*





Clone 36

ATCTTATKTCATACATtWTCTGCATHAAATTTAgCACATCACACTCCCTAAAACAmATAGCCAC
ATGWAMACAAYCCACTTCACATTTATACACCAAATTCTAGCTGCMRCATATKWKCGAATCTR
CCSCTCTDGATCGGTGAGGGCTGGGBGATCGATCCACGGCTAGGGCBTGTAACACGCWAGCA
CRATGATATTGATCTGACGTTTCCCGGCGAGAGGCGCGGCGAGCTCGAGGCAATGGTTGAAAT
GCTCGGCGGGCGCGWTCACGGAGGAGTTGGACTATGGATTCTTAGCGGAGATCSGGGATGAG
TTACTTGACTGCGAACCTGCTTGGTGGGCAGACGAAGCGTATGAAATCGCGGAGGCTTCCGCA
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TGYGTGCCGCTTTCAGGTCGCGATATGCGGCCTAACAATTCGTCCAAGTCGACGCCGCTTCBG
CGGGCKTTTAACTCAMGCGTTAGATGCCCTAAGCMCATAATTGCTCACAGCCAAaMTATCAR
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MGAAAWTKYKGKTKKRgTGTCATTARGRGATTTAaRCHGMAHKTBNBGGTTTGRKYYCCTTA
DRVRVACTWACCHSAAWKNBGKCTKGDTGCCTAWRRVRACHWWACCGCACWTBCSBbBTKG
GTGCYHWAGRRRCTTHACMCAHTTCGBGCKTGKKKSYCTARAVRRCATAMaSACAtYTHGTBY
YGTGTGBCyHACRVGCaYWCCG

COMMERCIAL SEQUENCING EQUIPMENTS