DEVELOPMENT AND APPLICATION OF REAL-TIME PCR METHODS FOR THE DETECTION AND TYPING OF EXTENDED-SPECTRUM b-LACTAMASES IN CLINICAL ISOLATES OF *ENTEROBACTERIACEAE*

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РАЗРАБОТКА И ПРИМЕНЕНИЕ МЕТОДОВ ПЦР В РЕЖИМЕ РЕАЛЬНОГО ВРЕМЕНИ ДЛЯ ИДЕНТИФИКАЦИИ b-ЛАКТАМАЗ РАСШИРЕННОГО СПЕКТРА У КЛИНИЧЕСКИХ ШТАММОВ ЭНТЕРОБАКТЕРИЙ

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EXTENDED-SPECTRUM b-LACTAMASES (ESBLs)

- Active against all b-lactams (penicillins, I-IV gen. cephalosporins, aztreonam) except cephamycins and carbapenems
- (-¯) Inhibited by active-site directed inhibitors (clavulanic acid, sulbactam, tazobactam)
- Ambler molecular class **A** or class **D** (active site serine b-lactamases)
- Bush, Jacoby and Medeiros functional groups: **2be**, **2d**, **2f**
- Multiple genetic types: **CTX-M**, **SHV**, **TEM**, etc.
- Typically plasmid-encoded, often associated with mobile elements
- Produced mainly by *Enterobacteriaceae*

WHY ESBLs ARE IMPORTANT?

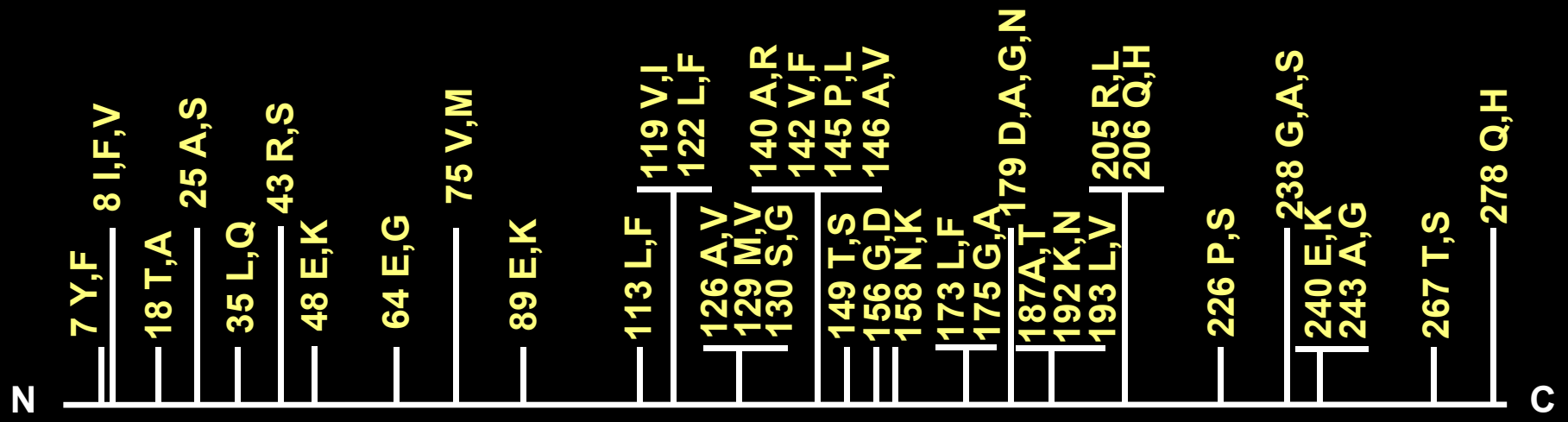
- **Confer resistance to the most commonly used antibiotics** which constitute the first-line therapy for nosocomial infections
- Difficult to detect by routine susceptibility tests:
  FALSE susceptible ESBL producers are a **common cause of treatment failures with modern cephalosporins**
- Complex epidemiology:
  may rapidly spread by clonal transmission or plasmid transfer
- Frequently associated with resistance determinants to non-b-lactam agents (fluoroquinolones, aminoglycosides, tetracyclines, sulfonamides, etc).
  ESBL producers are often multiply resistant

MOLECULAR TYPING OF ESBLs

- Provides a basis for understanding the epidemiology and evolution of ESBLs
- There is no one single molecular test to detect all ESBL types
- Numerous methods have been proposed for rapid identification of ESBLs that belong to certain genetic groups (e.g. TEM, SHV, CTX-M):
  - Spoligotyping;
  - PCR-SSCP;
  - minisequencing;
  - PCR-RFLP;
  - LCR;
  - real-time PCR
- But none of these techniques can identify all members of any group in a single step

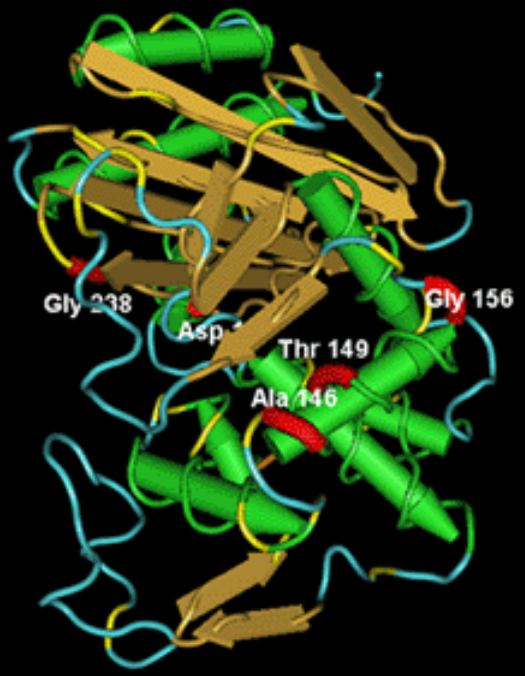
THE SHV-TYPE ESBLs

- SHV-1 penicillinase (»32 kDa) is a direct progenitor
  - species-specific (chromosomally encoded) in *Klebsiella pneumoniae*
  - often plasmid mediated in different species of *Enterobacteriaceae*
- SHV ESBLs evolve by acquisition of point mutations
- Over 60 amino acid variants currently exist*



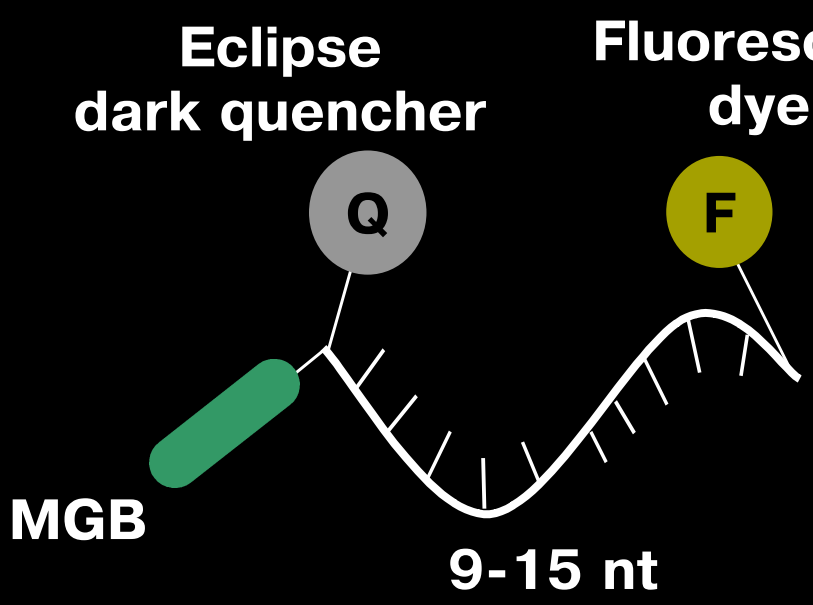
* http://www.lahey.org/studies/webt.htm

THE SHV-TYPE ESBLs



Substitutions at only a few amino acid residues are required for extended-spectrum activity

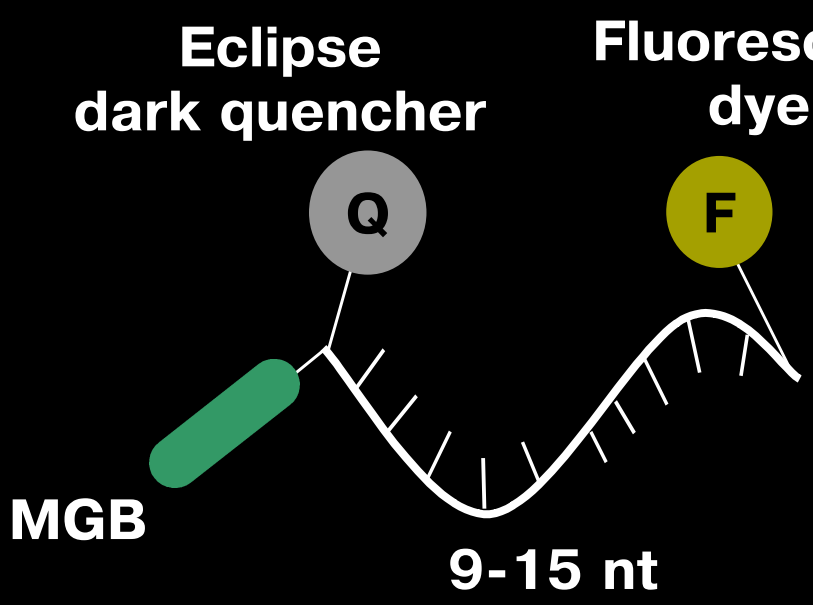
MGB ECLIPSE™ PROBES*



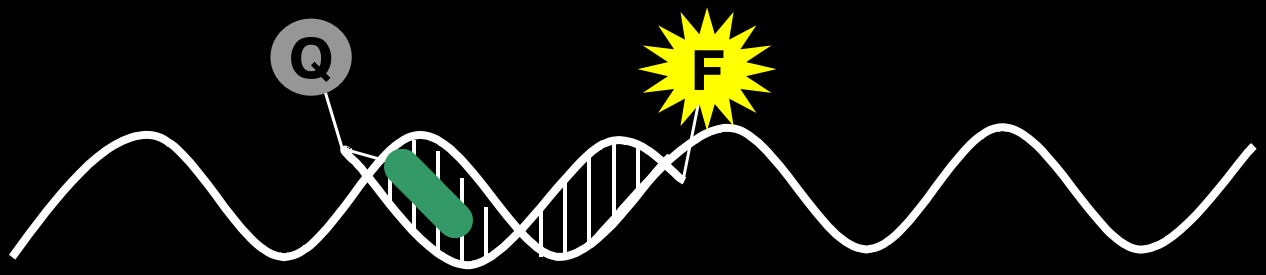
- Contain minor grove binder (MGB) and Eclipse dark quencher at 5’ end
- Contain fluorescent dye at 3’ end
- Short probes(9-15 nt) with high Tm and precise binding to target

** Epoch Biosciences (Bothell, WA, USA)*

MGB ECLIPSE™ PROBES*

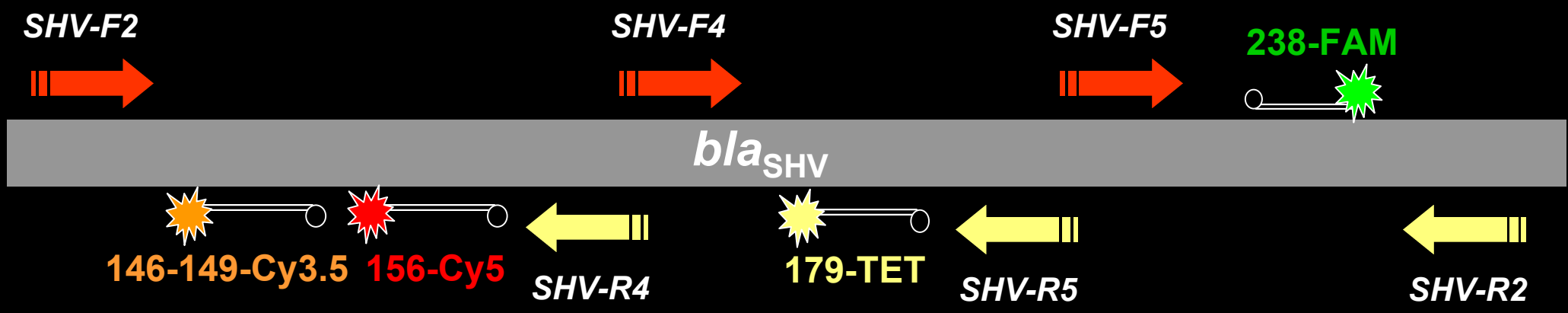


- Quenched in random coil state
- Fluorescing when bond to target
- 5'-nuclease resistant Þ suitable for postamplification meting curve analysis



* *Epoch Biosciences (Bothell, WA, USA)*

DESIGN OF MULTIPLEX SINGLE-TUBE PCR AND MELTING CURVE ANALYSIS FOR DETECTION OF SHV ESBLs



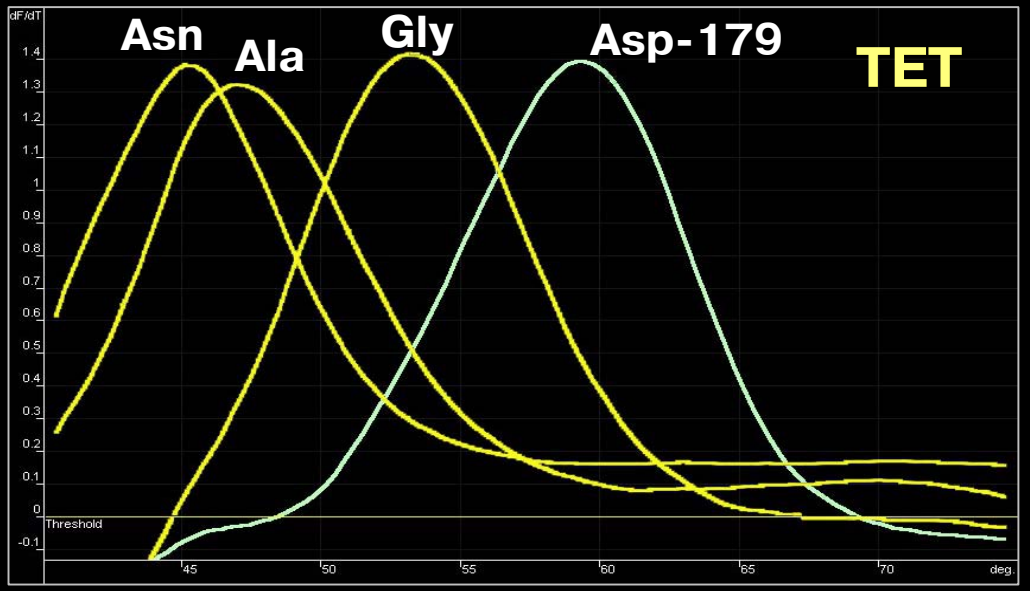
- 4 differentially labelled probes complementary to WT sequences at key mutation sites
- Each mutation specifically shifts the Tm of the corresponding probe
- Asymmetric primer ratio to favour formation of the strands complementary to the probes

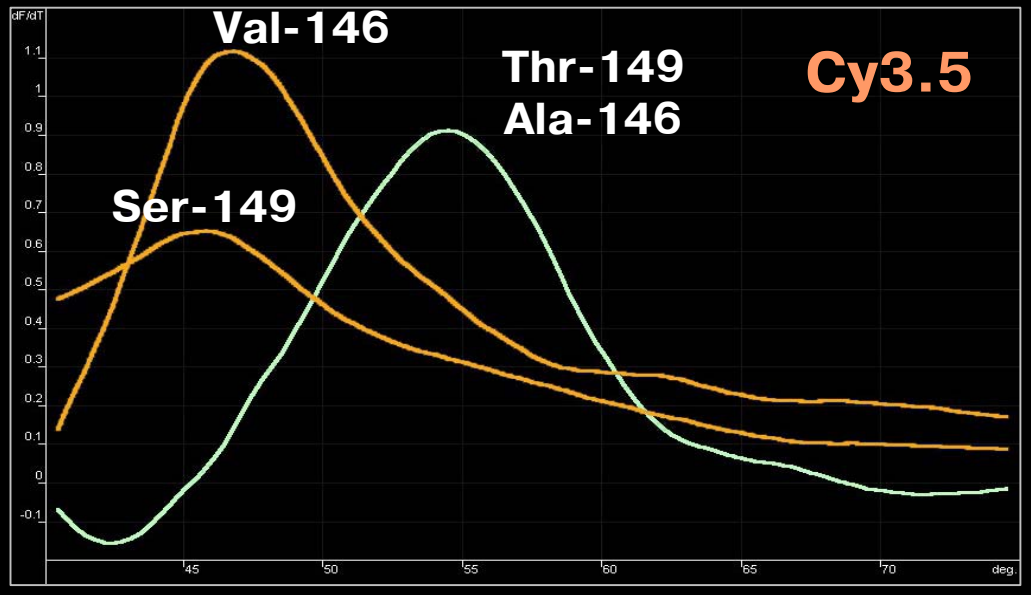
A.Ekimov et al., 14th ECCMID, 2004, P944

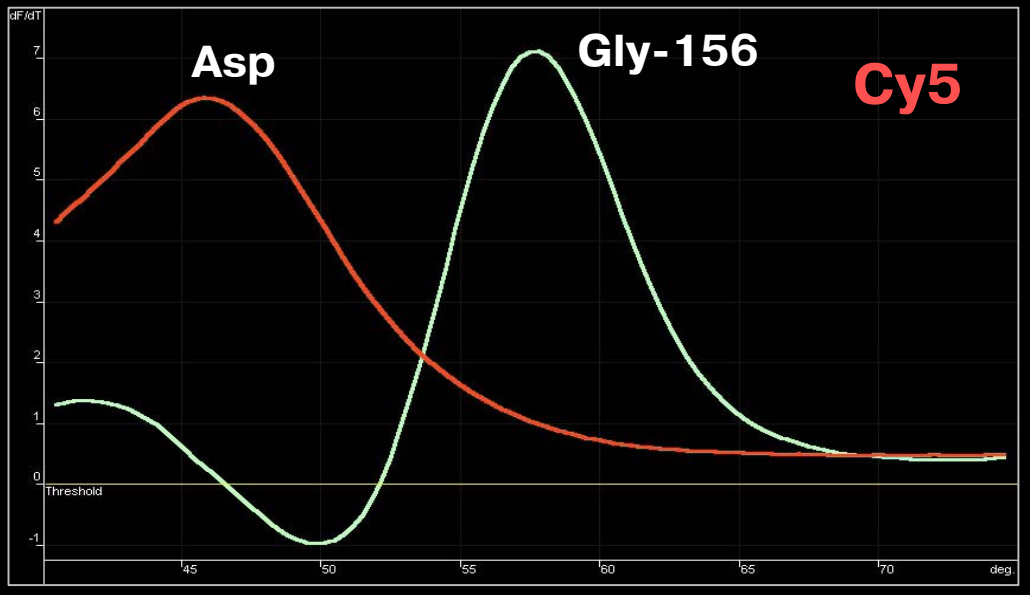
CONTROL STRAINS WITH KNOWN MUTATIONS IN SHVs

Mutation	Detected with probe
WT, non-ESBL control (SHV-1)	All probes
Ser-238 (SHV-2, -3, -4)	238-FAM
Ser-238 + Lys-240 (SHV-5)	238-FAM
Ala-238 (SHV-18)	238-FAM
Ala-179 (SHV-6)	179-TET
Asn-179 (SHV-8)	179-TET
Gly-179 (site-directed mut.)	179-TET
Asp-156 (site-directed mut.)	156-Cy3.5
Ser-149 (site-directed mut.)	146-149-Cy5
Val-146 (site-directed mut.)	146-149-Cy5

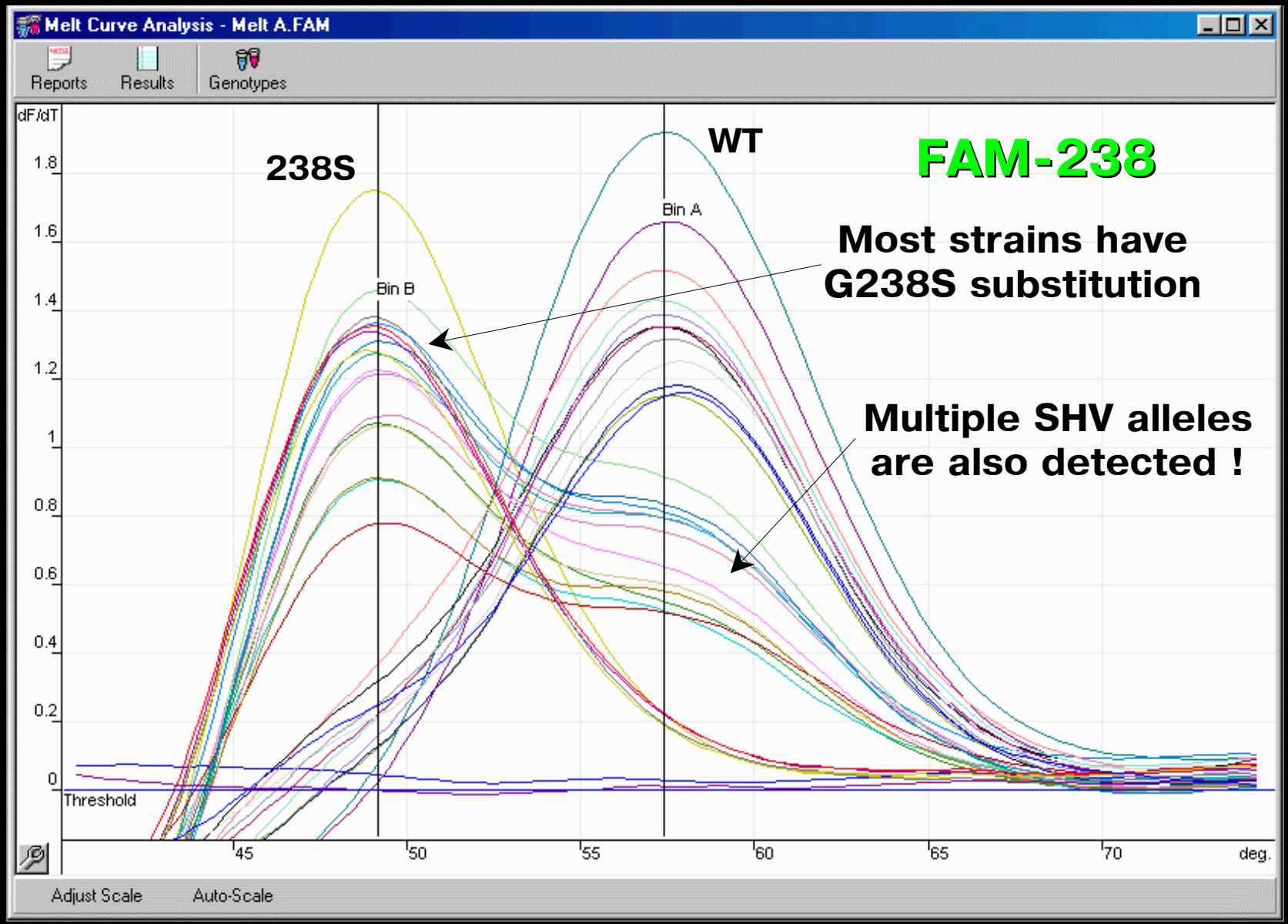
DETECTION AND DIFFERENTIATION OF ALL THE KNOWN ESBL MUTATIONS BY MELTING CURVE ANALYSIS





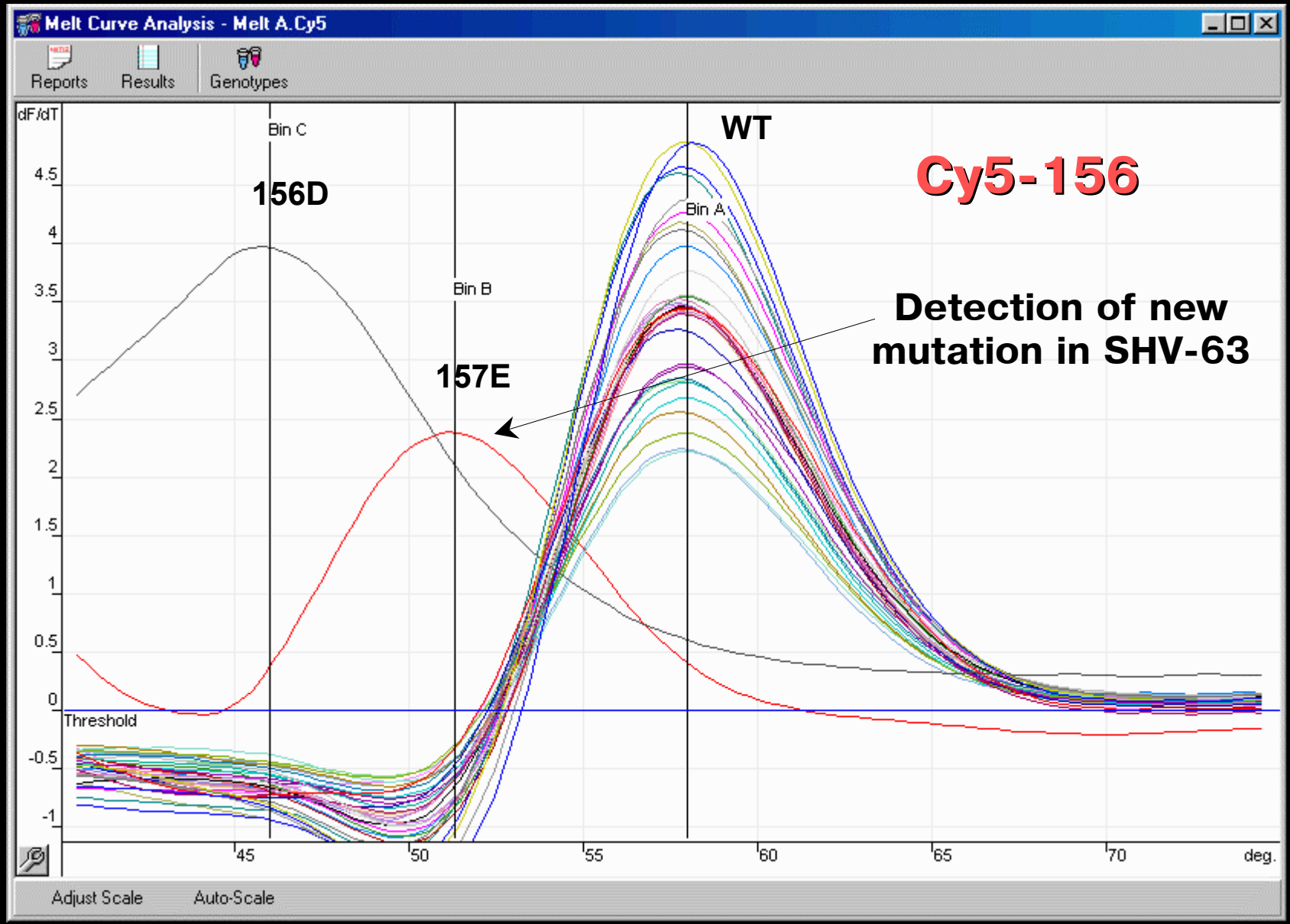


ANALYSIS OF CLINICAL ISOLATES



Rotor-Gene 2000 Real-Time PCR System

ANALYSIS OF CLINICAL ISOLATES



Rotor-Gene 2000 Real-Time PCR System

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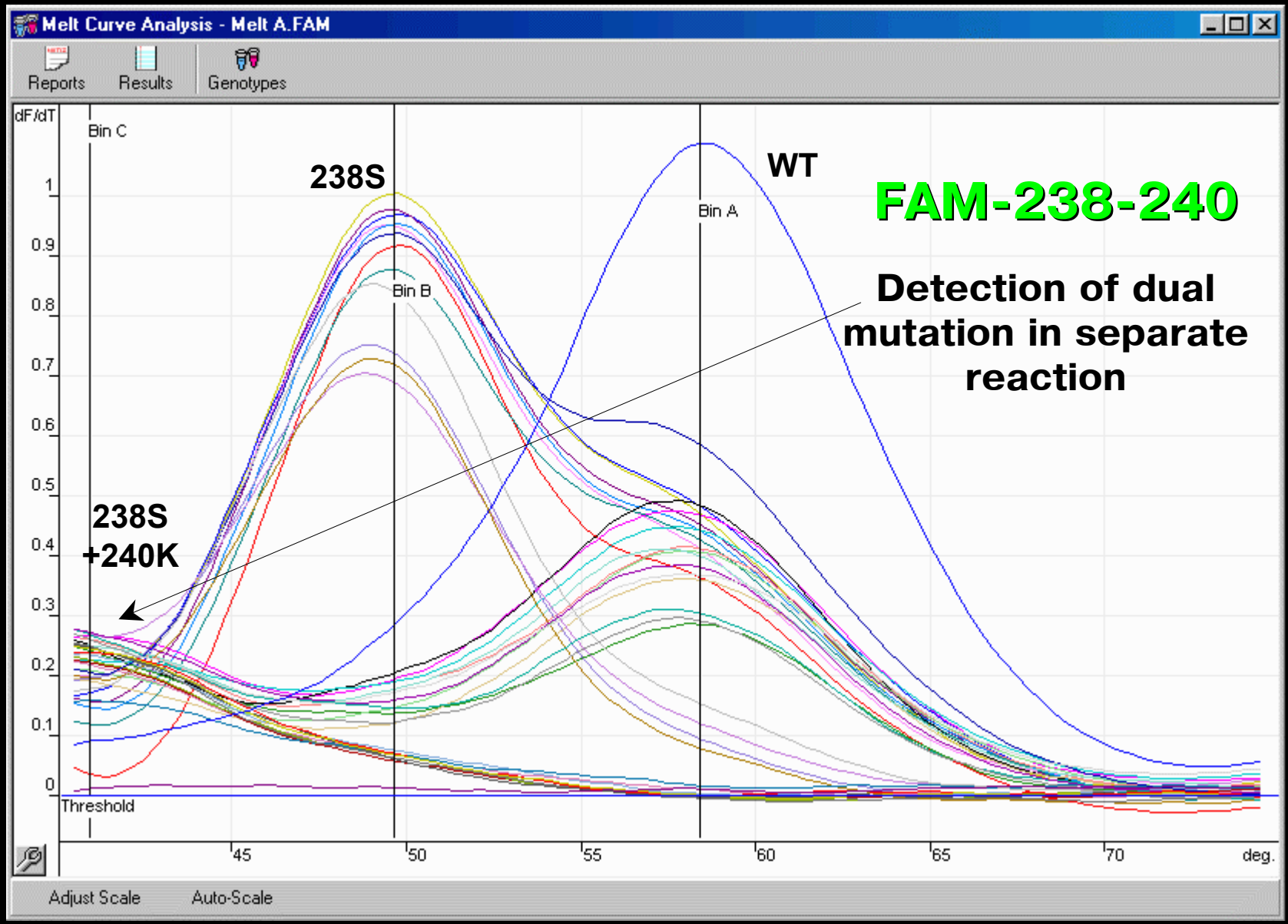
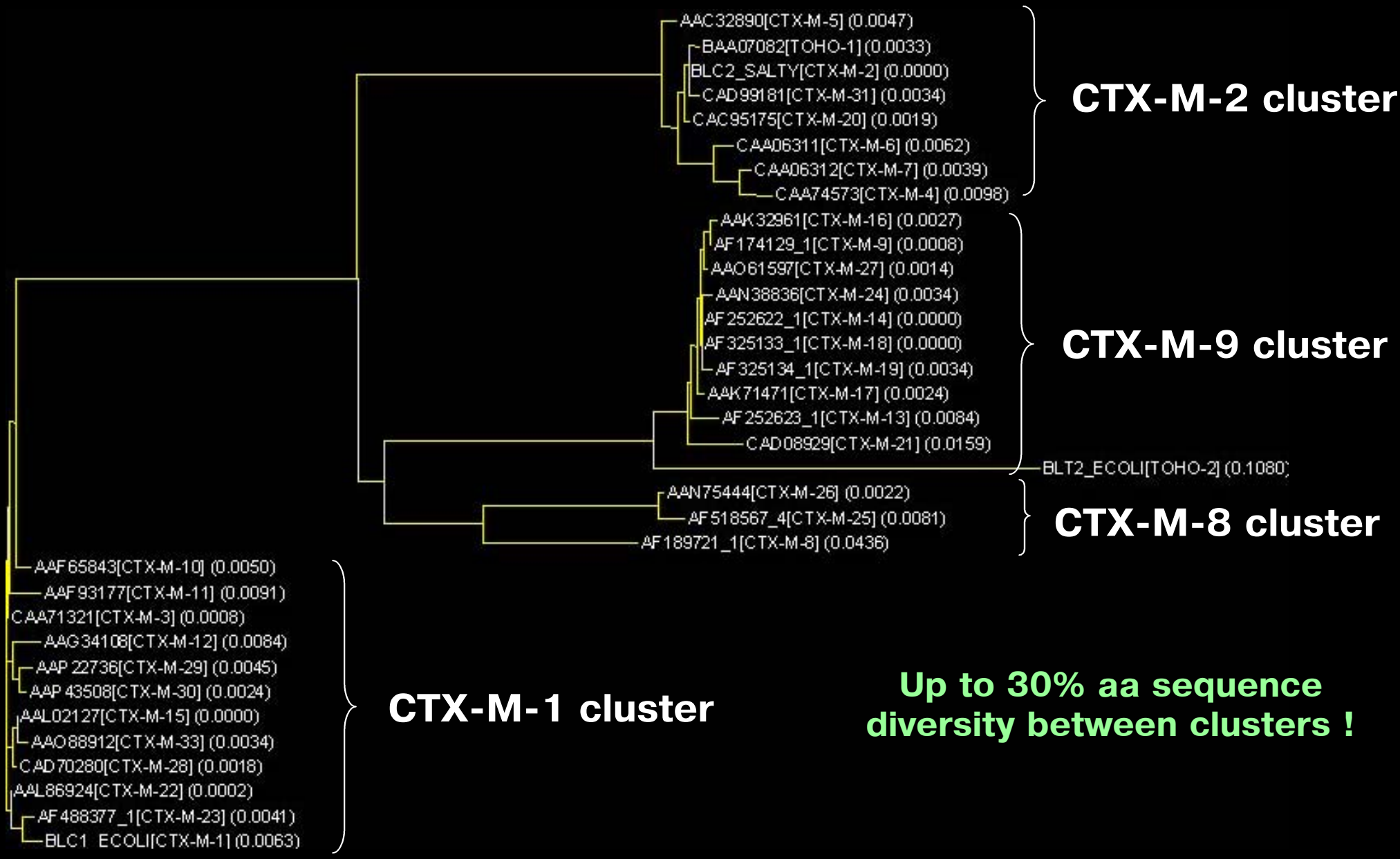


Rotor-Gene 2000 Real-Time PCR System

DETECTION OF SHV ESBLs BY MULTIPLEX REAL-TIME PCR WITH MGB ECLIPSE™ PROBES

- All the known mutations conferring extended-spectrum activity on SHV b-lactamases can be detected and discriminated in a single reaction
- To our knowledge, this is the first example of detection of 8 mutations in 5 codons using a single-tube real-time PCR
- New mutations at the key codons can also be identified
- Plasmid-mediated SHV ESBLs can be detected on the background of chromosomally-encoded SHV-1 in *K.pneumoniae*
- Extremely fast and processive
  (< 4 h for DNA isolation, PCR and melting curve analysis with 33 strains without opening the tube)
- No risk of contamination by PCR products

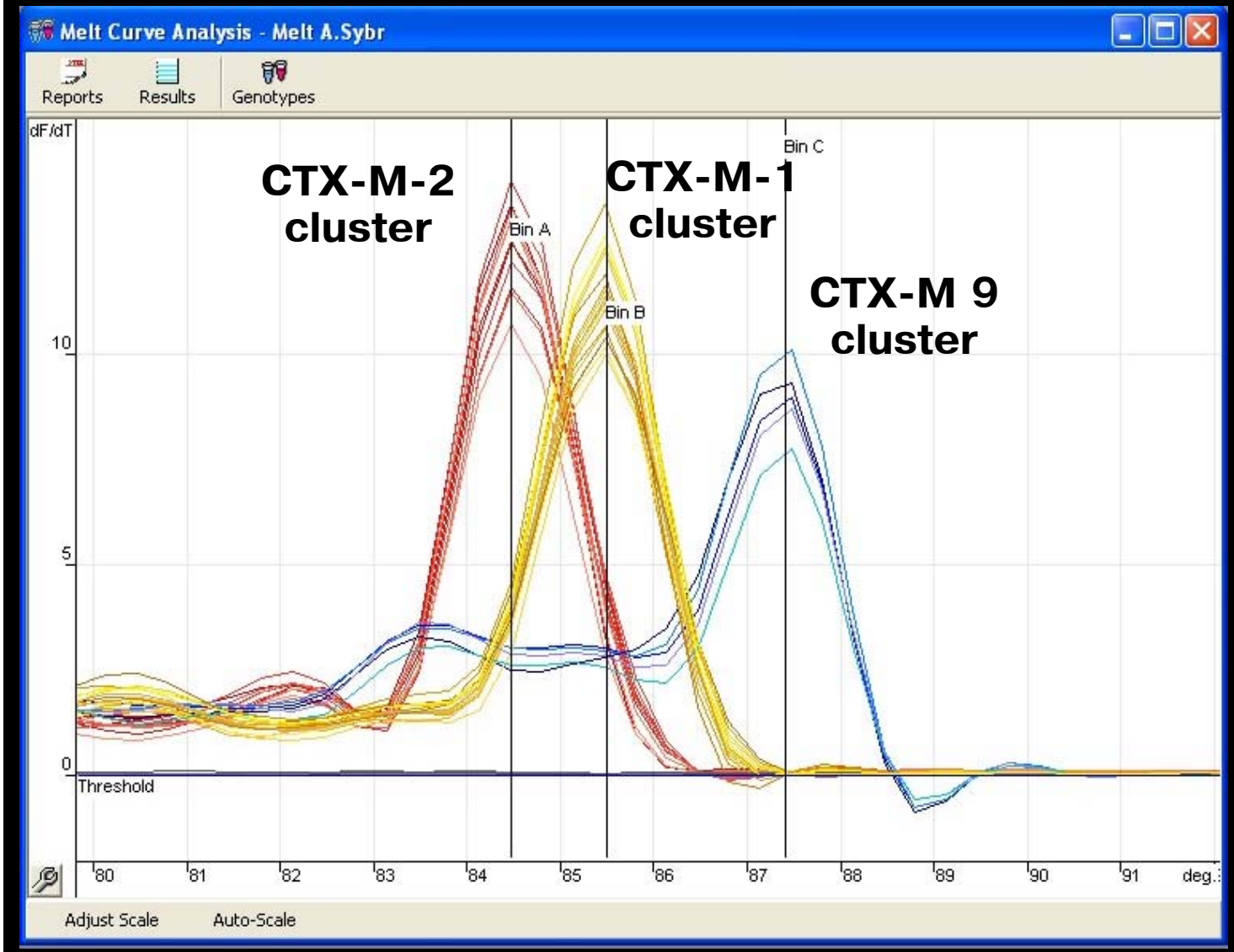
THE CTX-M-TYPE ESBLs



Up to 30% aa sequence diversity between clusters !

http://www.lahey.org/studies/other.asp

REAL-TIME PCR WITH UNIVERSAL CTX-M PRIMERS AND SYBR GREEN I



- Primers were designed to amplify 519bp PCR-product from all the subtypes
- Members of different clusters can be distinguished by melting curve analysis

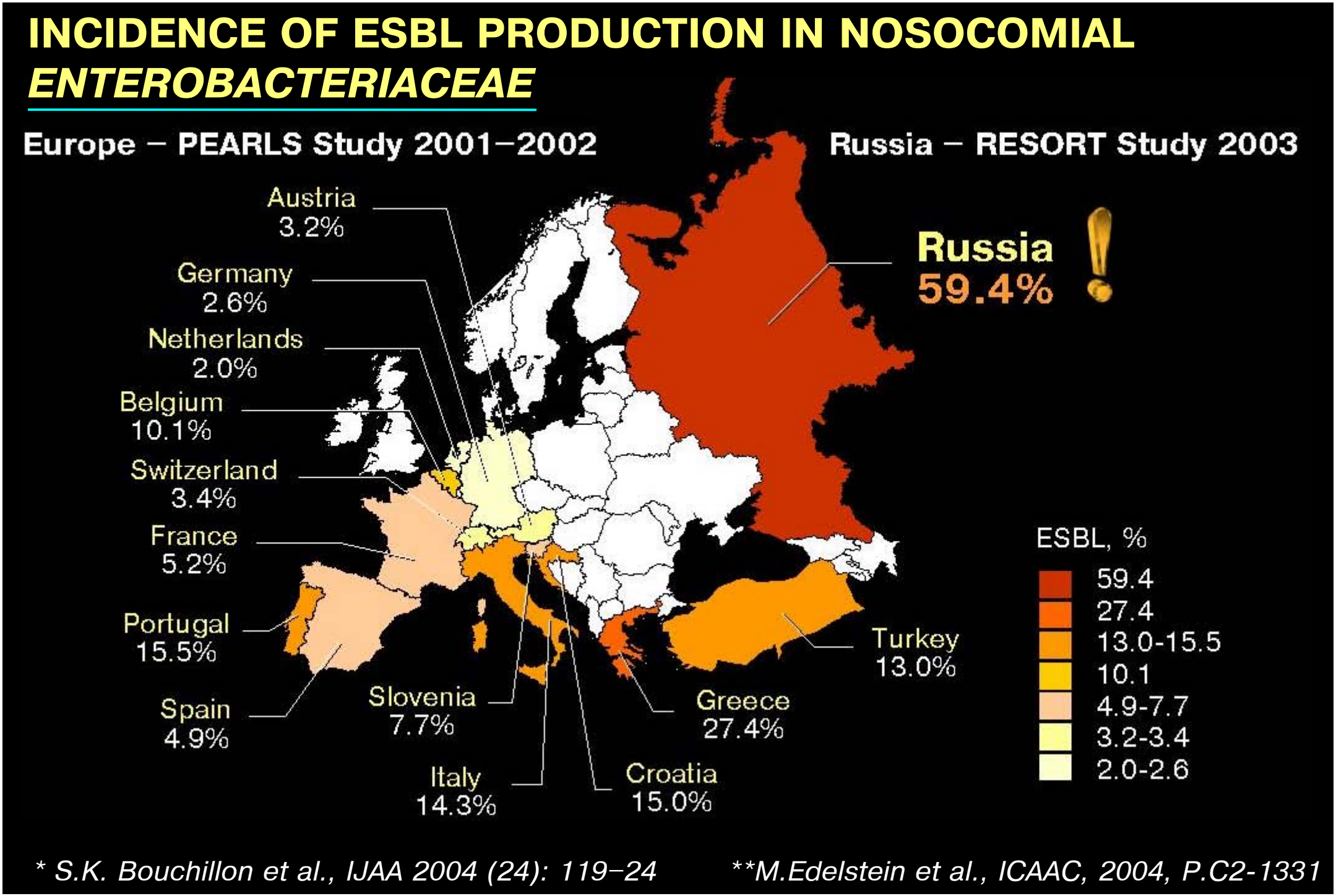
M.Edelstein et al., 15th ECCMID, 2005, P. 649

INCIDENCE OF ESBL-PRODUCING *ENTEROBACTERIACEAE* IN RUSSIAN ICUs

Data from RESORT study - 2003
648 strains from 33 hospitals of 22 cities

M.Edelstein et al., ICAAC, 2004, P.C2-1331

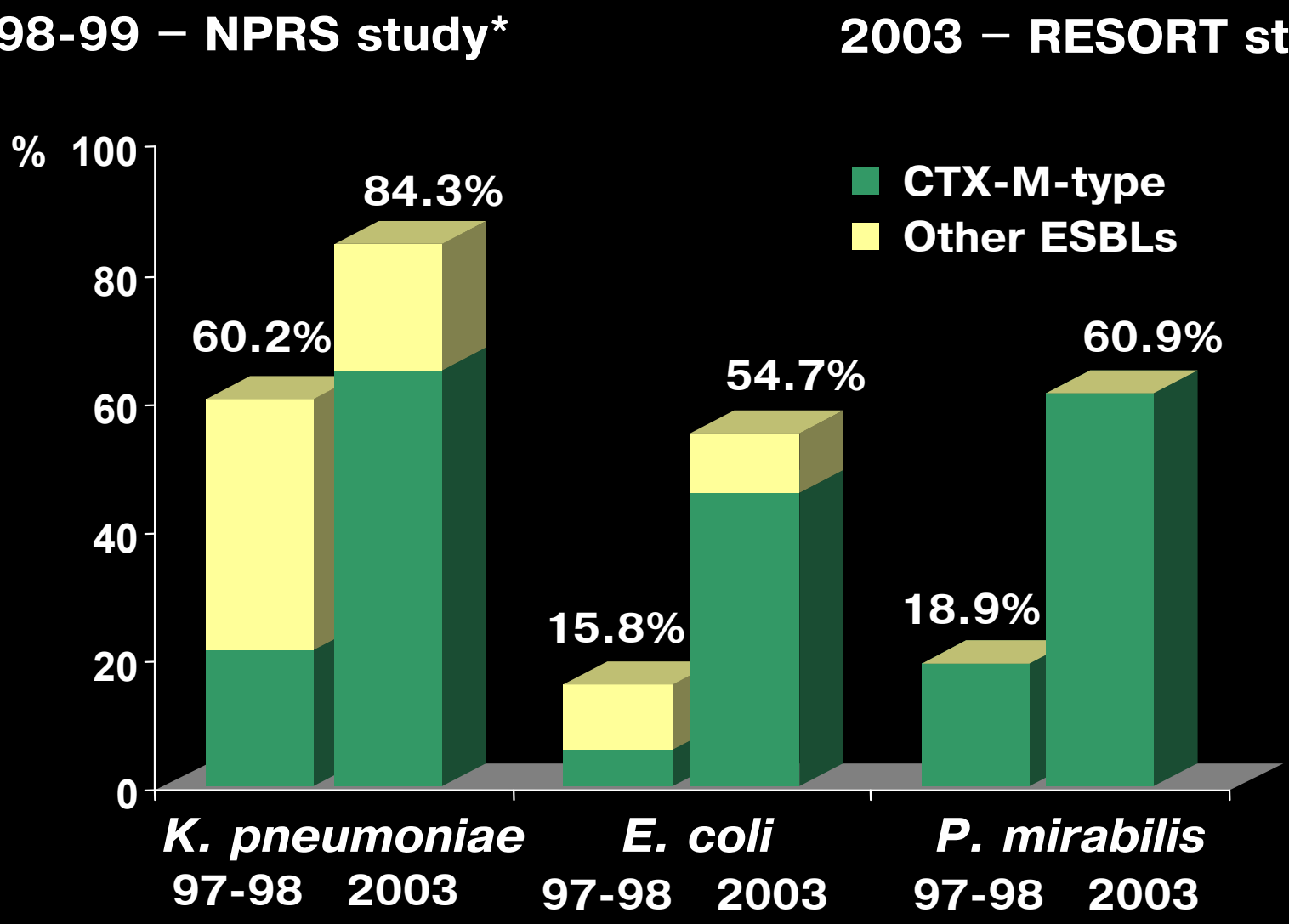
INCIDENCE OF ESBL PRODUCTION IN NOSOCOMIAL *ENTEROBACTERIACEAE*



** S.K. Bouchillon et al., IJAA 2004 (24): 119–24*

***M.Edelstein et al., ICAAC, 2004, P.C2-1331*

DRAMATIC INCREASE IN THE PROPORTION OF CTX-M ESBLs IN RUSSIA

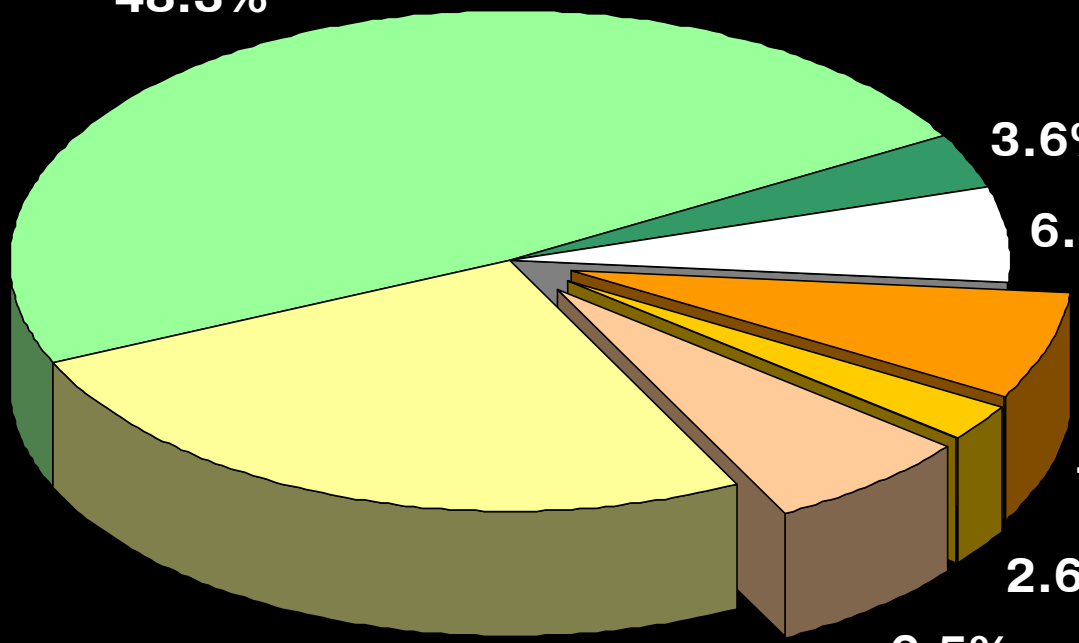


* M.Edelstein et al., AAC 2003 (47): 3724–32

** M.Edelstein et al., ICAAC, 2004, P.C2-1331

SPECIES DISTRIBUTION AMONG ESBL-PRODUCING *ENTEROBACTERIACEAE*

RESORT study – 2003
ESBL-producing strains (n=384)



K. pneumoniae 48.3%

3.6% *P. mirabilis*

6.0% Other species

7.0% *S. marcescens*

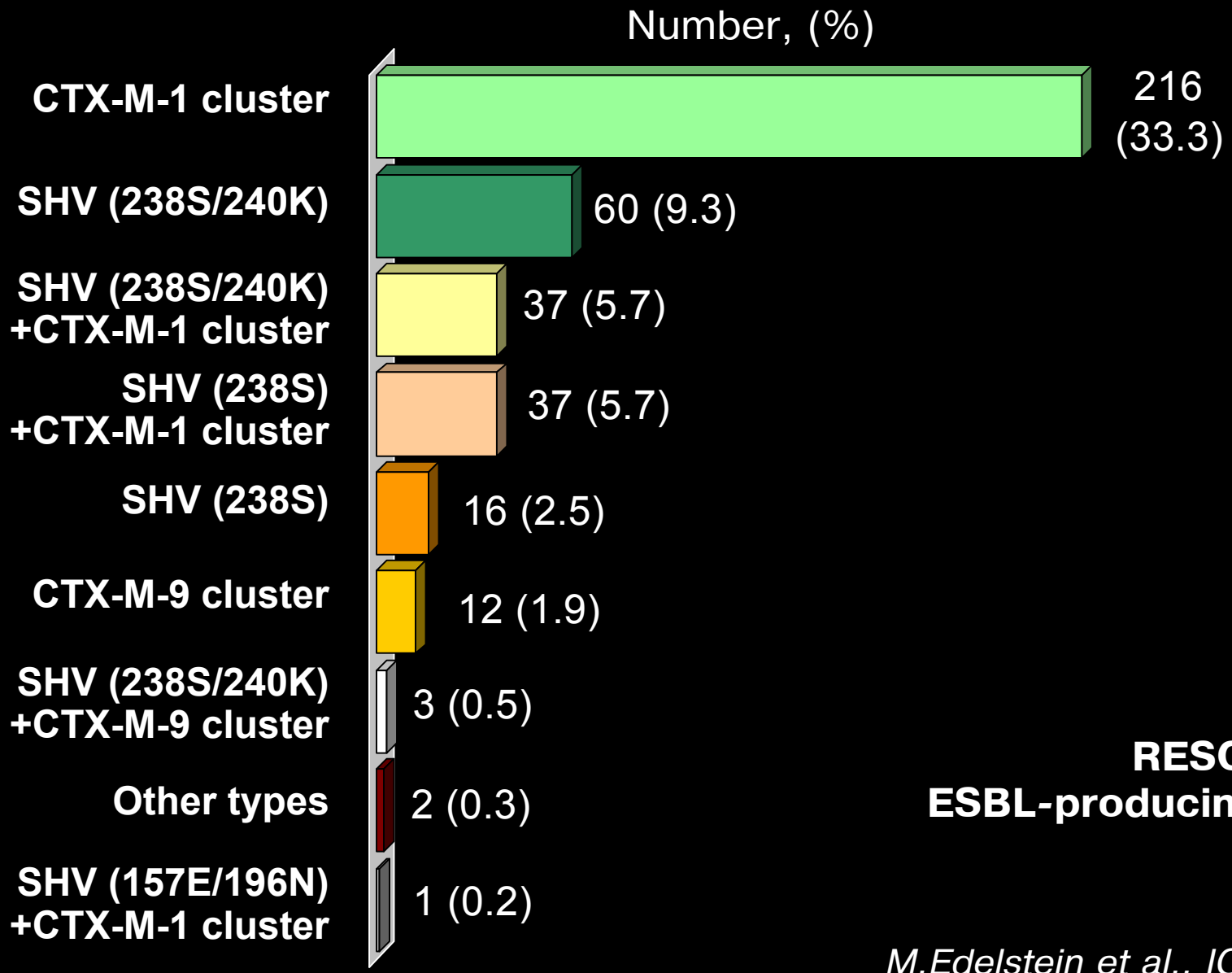
2.6% *Citrobacter* spp.

6.5% *Enterobacter* spp.

26.0% *E. coli*

M.Edelstein et al., ICAAC, 2004, P.C2-1331

PREVALENCES OF DIFFERENT ESBL TYPES AND THEIR COMBINATIONS IN RUSSIAN NOSOCOMIAL ISOLATES



RESORT Study – 2003
ESBL-producing strains (n=385)

M.Edelstein et al., ICAAC, 2004, P.C2-1331

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