

EUROPEAN PROSPECTIVE STUDY OF *CLOSTRIDIUM  
DIFFICILE* STRAINS: PHENOTYPIC AND GENOTYPIC  
CHARACTERIZATION OF THE ISOLATES FROM  
DIFFERENT CLINICAL STATUS : INTERIM RESULTS

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for the European Study Group on *Clostridium difficile*  
(ESGCD)

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# OBJECTIVES

## Main objectives :

- To establish a well defined European collection of *C. difficile* strains
- To study and to compare the phenotypic and genotypic markers of isolates (*prevalence of toxinotype III ?*)
- To get an estimation of the incidence of *C. difficile* infections

## Other objectives :

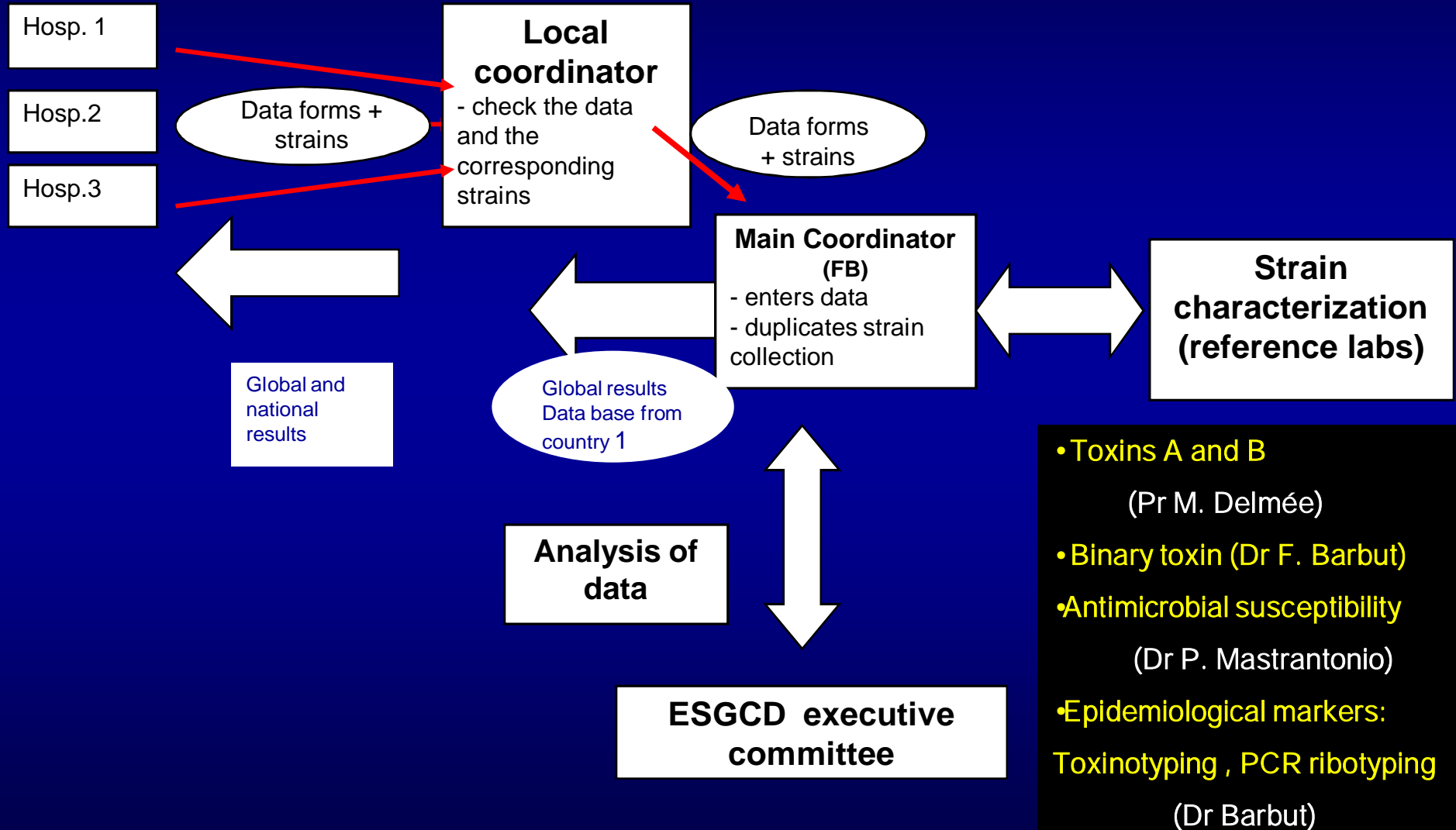
- To correlate clinical presentations with phenotypic and genotypic features *C. difficile*.
- To get the baseline characteristics of *C. difficile* strains isolated in 2005 making further investigations possible to follow trends in antimicrobial susceptibility, serogroups, genotypic patterns...

# MATERIALS AND METHODS

- Design
  - Prospective European-wide study on *C. difficile* strains isolated during a 2-month period with phenotypic and genotypic characterization of isolates
- Requirements for participating hospitals:
  - to have an on-site laboratory of microbiology
  - to systematically perform culture each time a test for *C. difficile* is requested.
  - to be able to detect toxins A or B from stools or from strains
  - to fill out a questionnaire (=data form) for each isolated CD strain (whatever the toxin result is)

# GENERAL ORGANIZATION OF THE STUDY

## COUNTRY 1



# MATERIALS AND METHODS

- **Inclusion criteria:**

All the *C. difficile* strains (including the non toxigenic strains) isolated from inpatients (with community or nosocomial diarrhea)
- **Exclusion criteria:**

Strains from **outpatients** or **day-care patients**.  
Strains from **children** under 2 years old
- **Length of the study :**

a 2 month-period (or 1 month for every hospital which reaches 30 *C. difficile* strains at the end of the first month) between April and June.

# MATERIALS AND METHODS

- Clinical data form :
  - Clinical symptoms
  - Biological parameters
  - Risk factors (antimicrobial treatment, gastrointestinal procedures, previous hospitalization .....)
  - Endoscopic or radiologic examinations
  - Treatment and outcome

# MATERIALS AND METHODS

- Phenotypic detection of toxins A and B

(Pr M. Delmée, J. Van Broeck)

- Toxin A:

- *C. difficile* toxin A (Oxoid, UK)
    - suspension of 10 colonies in PBS

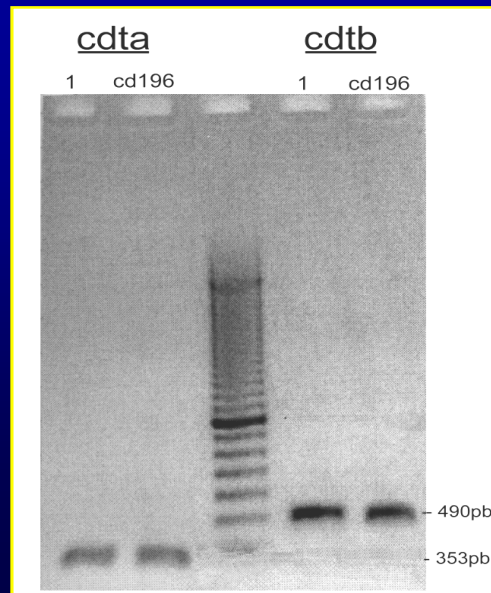
- Toxin B

- Cytotoxicity assay (Vero cells)
    - Supernatant of a BHI broth incubated 4 days

# MATERIALS AND METHODS

- Detection of binary toxin by PCR (Dr Barbut, L. Bonné, B. Burghoffer)
  - DNA extracted with Instagene\*
  - Primers (Stubbs et al., FEMS Microbiol. Lett 2000, 186, 306-607)

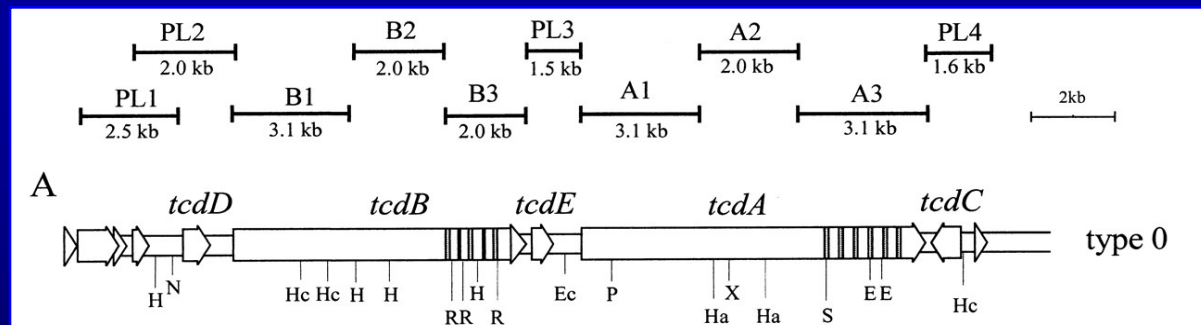
Cdta pos	5'-TGAACCTGGAAAAGGTGATG-3'	0.353 kb
Cdta rev	5'-AGGATTATTTACTGGACCATTTG-3'	
Cdtb rev	5'-ACCGGATCTCTTGCTTCAGTC-3'	
Cdtb pos	5'-CTTATTGCAAGTAAATACTGAG-3'	0.490 kb
  - CD 196 was used as positive control for detection of *cdtA* and *cdtB*.



**PCR of *cdtA* and *cdtB*  
(binary toxin)**

# MATERIALS AND METHODS

- **Toxinotyping (Dr Barbut, L. Bonné, B. Burghoffer)**
  - Method described by Rupnik et al., (JCM 1998, 36, 2240-47 and CMI 2001, 7, 417-420)
  - PCR-RFLP based method for analysing the polymorphism of the Paloc region compared to VPI 10463 as referred toxinotype 0
  - Amplification of A3 (A3C-A4N) and B1(B1C- B2N) fragments restricted by *EcoR1* (A3) and *Acc1* and *Hinc2* (B1)
  - Toxinotyping scheme : <http://mf.uni-mb.si/mikro/tox/>



- **PCR for *tcdC* (Van den Berg, 2005)**
  - *tcdC*for : 5' CATATCCTTCTTCTCCTCTTC-3' 159 bp
  - *tcdC*rev : 5' AATTGTCTGATGCTGAACC-3'

# MATERIALS AND METHODS

- **PCR ribotyping** (Dr Barbut, L. Bonné, B. Burghoffer)
  - Method described by Bidet et coll. (JCM 2000, 38, 2484-87)
  - Primers
    - CD1 : 5'-GTCCGGCTGGATCACCTCCT63'
    - CD2 : 5'- CCCTGCACCCTTAATAACTGGACC-3'
  - Electrophoresis through Resophor Agarose 3%
- **Antimicrobial susceptibility** (P. Mastrantonio, P. Spigaglia, F. Barbanti)
  - Etest method on Brucella blood agar supplemented with hemin and Vit K
  - Vancomycin (VA), Metronidazole (MZ), Erythromycin (EM), Clindamycin (CM), Moxifloxacin (MX), Tetracycline (TC)
  - Breakpoints used :
    - VA  $\geq 8$  mg/l; MZ  $\geq 8$  mg/l; MX  $\geq 4$  mg/l
    - TC  $\geq 8$  mg/l ; EM  $\geq 4$  mg/l; CM  $\geq 4$  mg/l

## PARTICIPATING COUNTRIES

14 countries, 38 hospitals, 486 strains

COUNTRY	Letter	No. Hospitals	Local coordinator	No. Strains
Belgium	A	3	M. Delmée	73
France	B	3	F. Barbut	53
Germany	C	3	G. Ackermann	86
Hungary	E	4	E. Nagy	48
GB	R	1	I. Poxton	9
Italy	F	3	P. Mastrantonio	27

COUNTRY	Letter	No. Hospitals	Local coordinator	No. Strains
Netherlands	G	3	E. Kuijper	29
Poland	H	3	H. Pituch	20
Spain	K	3	E. Bouza M. Somolinos	39
Switzerland	L	1	C. Balmelli	15
Sweden		3	M. Wult B. Andersson	22
Turkey	N	4	M. Yucesoy	9
Greece	P	3	H. Ladas	24
Ireland	D	1	D. Drudy	32

# Distribution of participating hospitals



# Strains and clinical data

- 486 strains and 464 clinical charts but an imperfect correspondance....
  - 30 clinical charts w/out strains
  - 43 strains w/out clinical charts
  - 435 complete files (strain + clinical charts)
- But ....
  - Some patients were children under 2 y.
  - Two countries sent a lot of repetitive isolates
- Therefore,
  - The data base needs to be cleaned
  - A last call for strains and clinical charts will be done

## Brief overview on clinical data (n=464)

- Age (excluding children < 2) :
  - 63±20 y. (68)
  - 57% of patients are older than 65 y.
- Sex :
  - male : 48.6%,
  - female : 51.4%
- Wards :
  - Med : 72.9%
  - Surgical : 18.6%
  - ICU : 8.5%
  - Obstetrics : 0 %
- Previous ATB : 76.4%
- Endoscopy : 11.9%
- PMC : 3.8%

# Brief overview on clinical data

Incidence varies widely (23 hosp.):  
 $2.45 \pm 1.8$  CDAD/10,000 patient-days  
(range : 0.14- 7.1)

Hospitals N :	0.13-0.14 CDAD/10,000 patient-days	
Hospital F:	0.5-1.2	«
Hospitals G, B:	2-7	«

USA :	12.1 CDAD /10,000 patients days (range 3-25.1)
(Sohn, ICHE 2005)	
USA :	5 /10,000 patient-days
(Archibald, CDC, JID 2004)	
Québec :	12.8 /10,000 patients-days
(INSPO report, 2005)	

# Toxigenicity

486 strains

414 toxigenic  
(85.2 %)

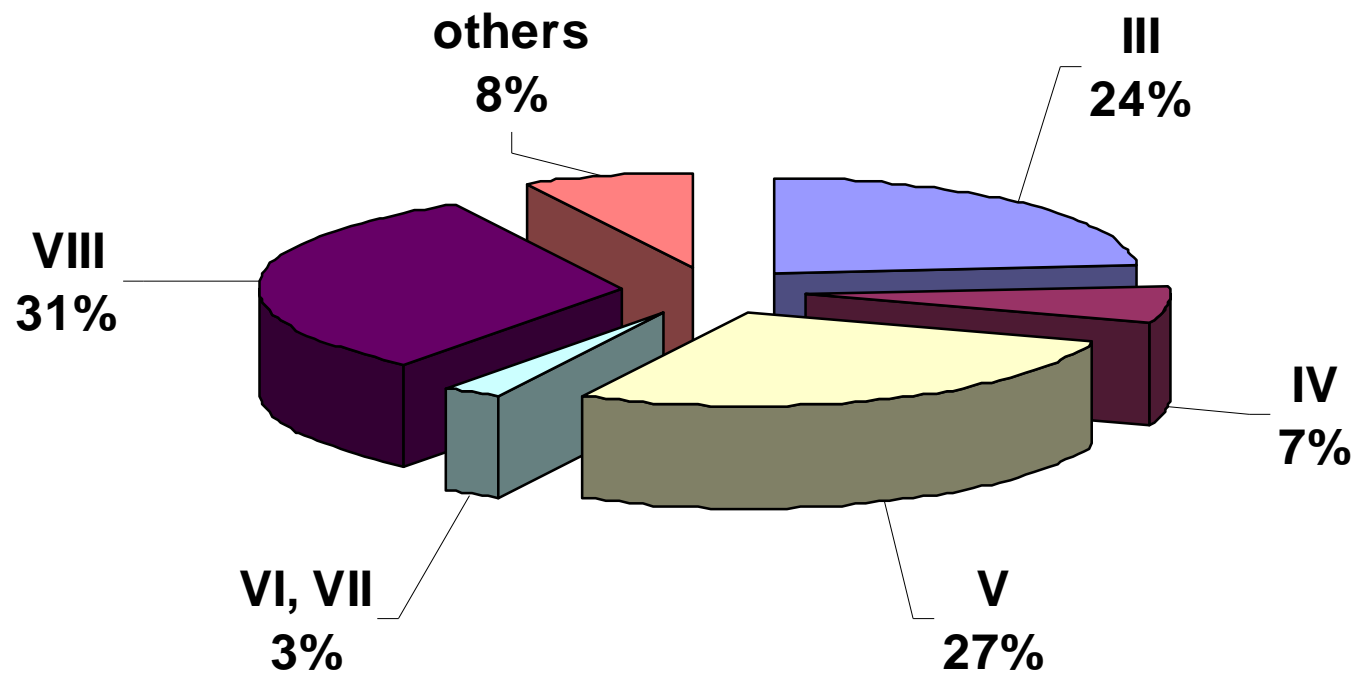
72 NON toxigenic  
(14.8 %)

Toxinotype 0  
308 (74.4%)

Toxinotype I-XXIV  
106 (25.6%)

*Geric B., JMM 2004: toxinotype = 12%*

# Distribution of toxin variant strains (n=106)



USA ( (Geric, JMM 2004) : III (17%), V (6%), VIII (12%)

## ANTIMICROBIAL SUSCEPTIBILITY

ATB	MIC50 (mg/l)	MIC90 (mg/l)	Range (min. max)	% R
VA	0.5	0.75	0.25-2	0
MZ	0.06	0.125	0.012-0.75	0
EM	1	>256	0.047->256	46.7
CM	4	>256	0.016->256	50.0
TC	0.032	0.38	0.023->32	8.8
MX	0.5	>32	0.032->32	33.9

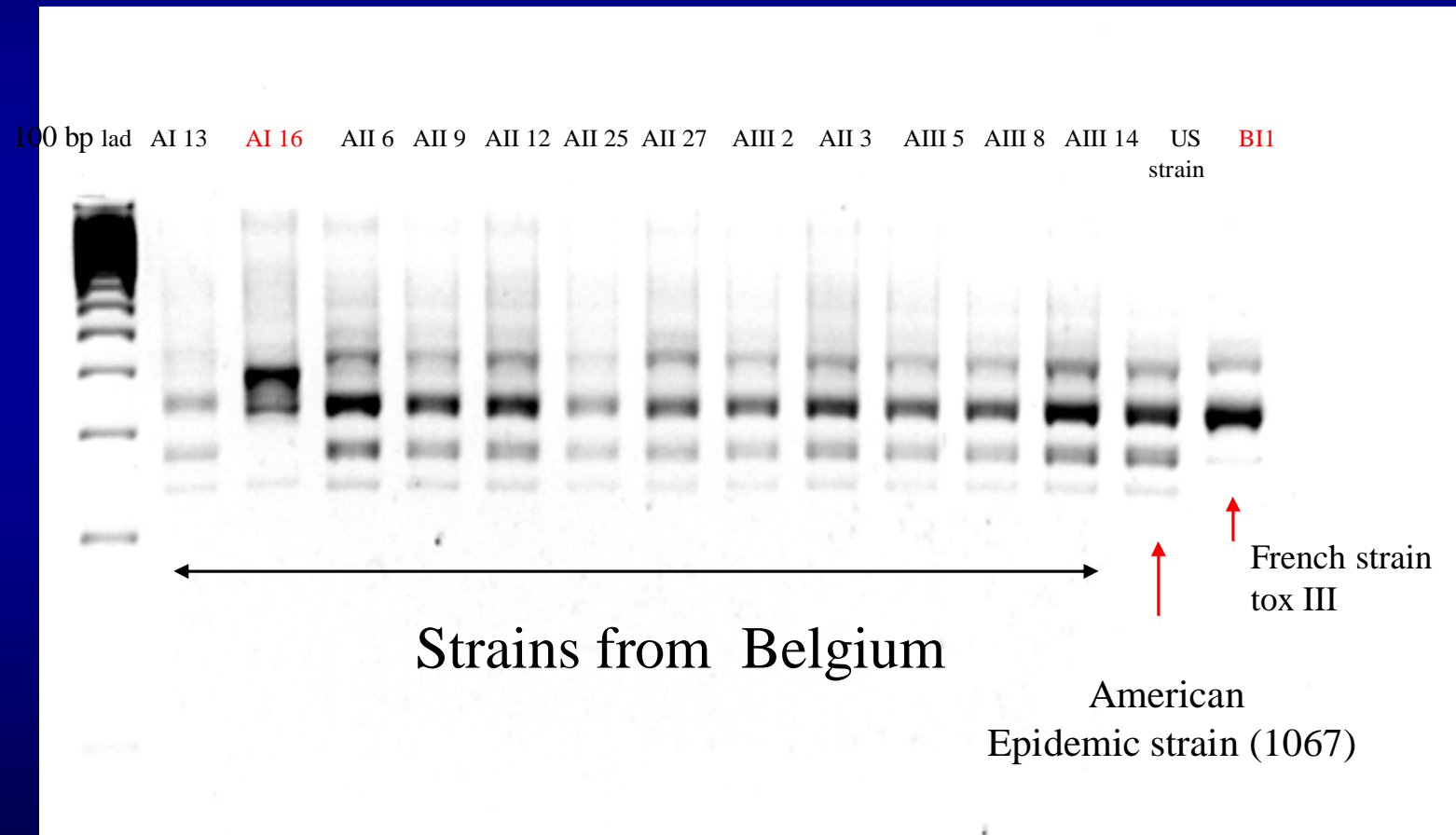
# ANTIMICROBIAL SUSCEPTIBILITY

Country	No. strains	% R EM+CM+TC+MX	% S EM+CM+TC+MX
Belgium	72	1.4	33.3
France	50	2	42
Germany	85	8.2	37.6
Ireland	31	9.7	32.3
Hungary	47	17	55.3
Italy	27	0	14.8
Netherlands	31	0	45.2
Poland	19	5.3	26.3
Spain	39	0	12.8
Switzerland	15	0	80
Sweden	22	0	81.8
Greece	23	8.7	21.7
Turkey	9	11.1	77.8
UK	8	0	12.5

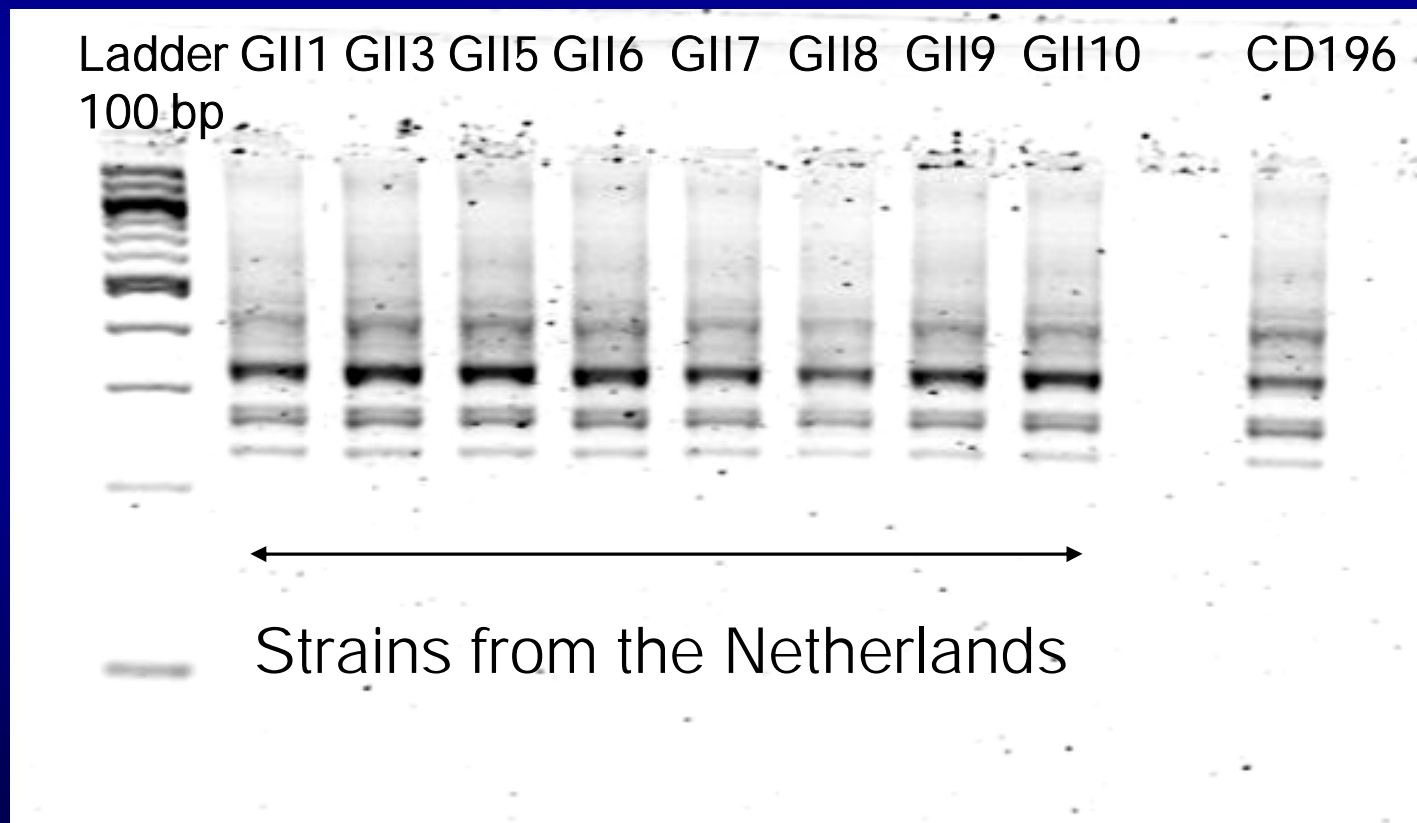
## Toxinotype III

- 25 strains isolated in 6 different countries
- 2 types of toxinotype III isolates by PCR-ribotyping :
  - « 027 » (n=20) : AI13, AII6, AII9, AI12, AII25, AII27, AIII2, AIII3, AIII5, AIII8, AIII14, GII1, GII3, GII5, GII6, GII7, GII8, GII9, GII 10, DI12
  - Non « 027 » (n=5) : AI16, KIII3, BI1, MI27, MI23
- Epidemic « 027 » strains were clustered in 2 countries (Netherlands and Belgium) and isolated once in Ireland.
- Non « 027 » toxinotype III isolates were found once in France, Belgium, Spain and Sweden (2 strains),

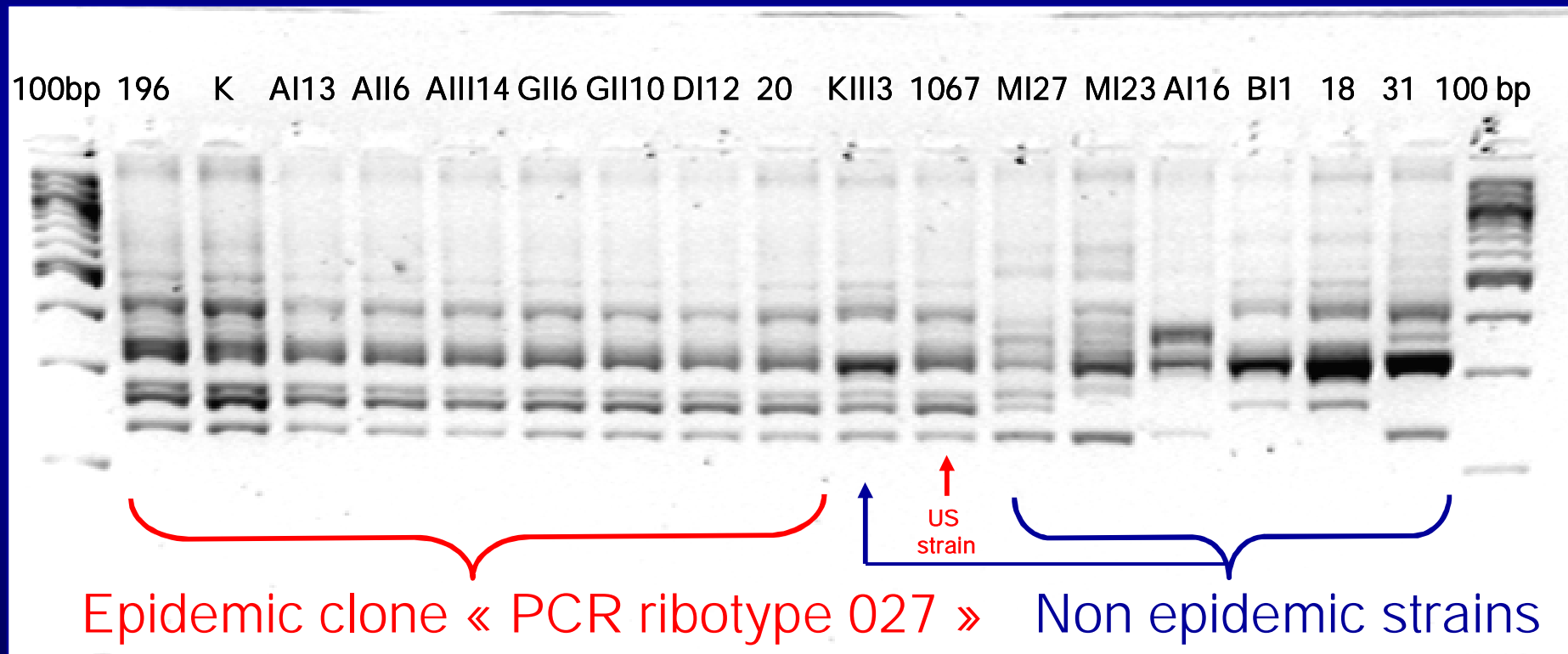
# PCR-ribotyping of toxinotype III isolates



# PCR-ribotyping of toxinotype III isolates



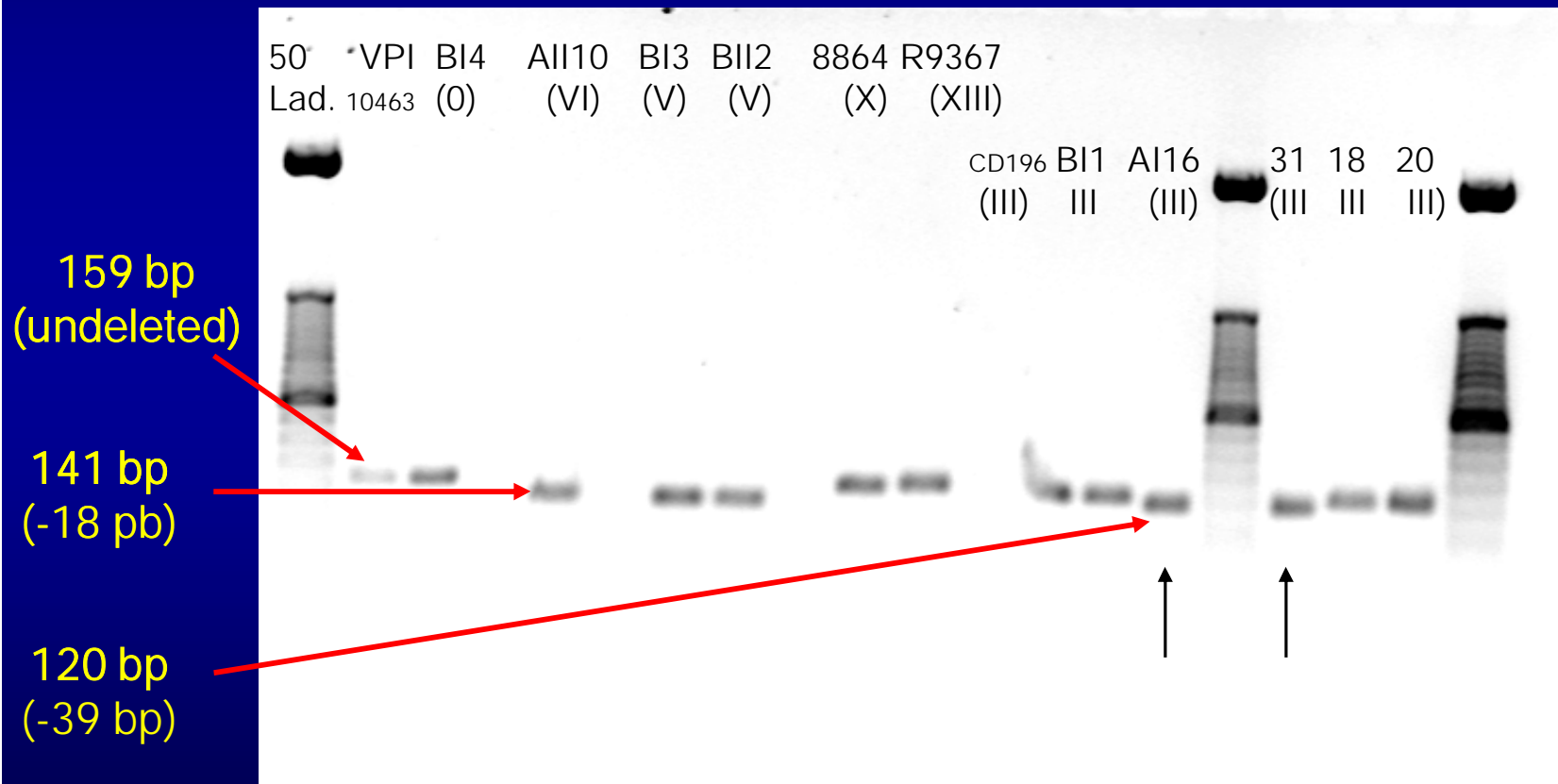
# PCR ribotyping of toxinotype III isolates



## Toxinotype III : antimicrobial susceptibility

- Epidemic «027» strains (n=20):
  - MIC EM > 256 mg/l
  - MIC MX  $\geq$  12 mg/l (except 1 strain with MIC= 6 mg/l)(AIII8)
- Non «027» toxinotype III strains (n=5) :
  - MIC EM = 0.064->256 mg/l (1 strain R)
  - MIC MX < 2 mg/l
- Resistance to MX is not specific of the epidemic clone : 31% of strains different from toxinotype III are resistant to MX

# *TcdC* polymorphism



## Toxinotype VIII

- 34 strains isolated in 6 countries
- All the strains were A-B+
- High prevalence in countries:
  - Poland (HII) : 80% (4/5)
  - Poland (HI) : 46.7 (5/11)
  - Ireland (DI) : 30.7% (4/13)
  - Sweden (MI) : 25% (3/12)
  - Germany (CIII) : 14.2% (7/49)
  - Ireland (DII) : 15.9% (3/19)
- PCR-ribotyping is under investigation

## Toxinotype V

- This toxinotype is widely distributed (9 countries)
- It is more prevalent in some countries :
  - France : 17% (9/53) (in 3 hospitals)
  - Greece : 29% (7/24) (in 2 hospitals)

## Binary toxin

- Binary toxin was found in 72 strains (17.4% of toxigenic strains)
- Binary toxin was not detected in non toxigenic strains

*Stubbs (Wales):* 6.4%

*Goncalves (France) :* 6%

*Rupnik (Asia) :* 1.6%

*Geric (USA) :* 6.1%

# Conclusion

- So far, the epidemic strain toxinotype III, PCR-ribotype « 027 » has been isolated in 3 countries
- In 4 hospitals, this clone represents 30% to 70% of all the toxigenic isolates
- Resistance to MX is not a good screening method for detecting the epidemic clone
- There is a polymorphism among toxinotype III strains

# PERSPECTIVES

- Correlation between toxinotypes and antimicrobial resistance patterns
- Analysis of clinical data : correlation between severity of disease and toxinotypes and/or binary toxin
- Correlation between previous antimicrobial treatment and antimicrobial susceptibility of isolates
- PCR ribotypes of toxinotype VIII and V
- ....

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