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C. difficile: application of molecular methods for diagnosis and epidemiological studies of CDAD

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- Molecular diagnostics for *C. difficile*
- Molecular typing methods for *C. difficile*
- Use of typing in epidemiological studies

Molecular diagnostic methods

- Direct detection in stool sample
- Characterization of isolated strain

Direct detection in stool samples

- PCRs
  several studies in early 90-ies

- Real time PCR
  Belanger et al., 2003
  tcdA and tcdB
  $5x10^4$ CFU/g feces

  van den Berg et al., 2006
  tcdB
  $1x10^5$ CFU/g feces

- detection of *C. difficile* <1h
- screening of asymptomatic carriers
  (situation: large outbreaks; studies; high local prevalence of infection)
- drawbacks:
  toxin gene – toxin
  time (?)
  strains for further investigation

Characterization of isolated strains

- Confirmation of species
  cdd3 (PaLoc downstream transporter gene)
  triose phosphate isomerase (*tpi*) Lemee et al., JCM 2004

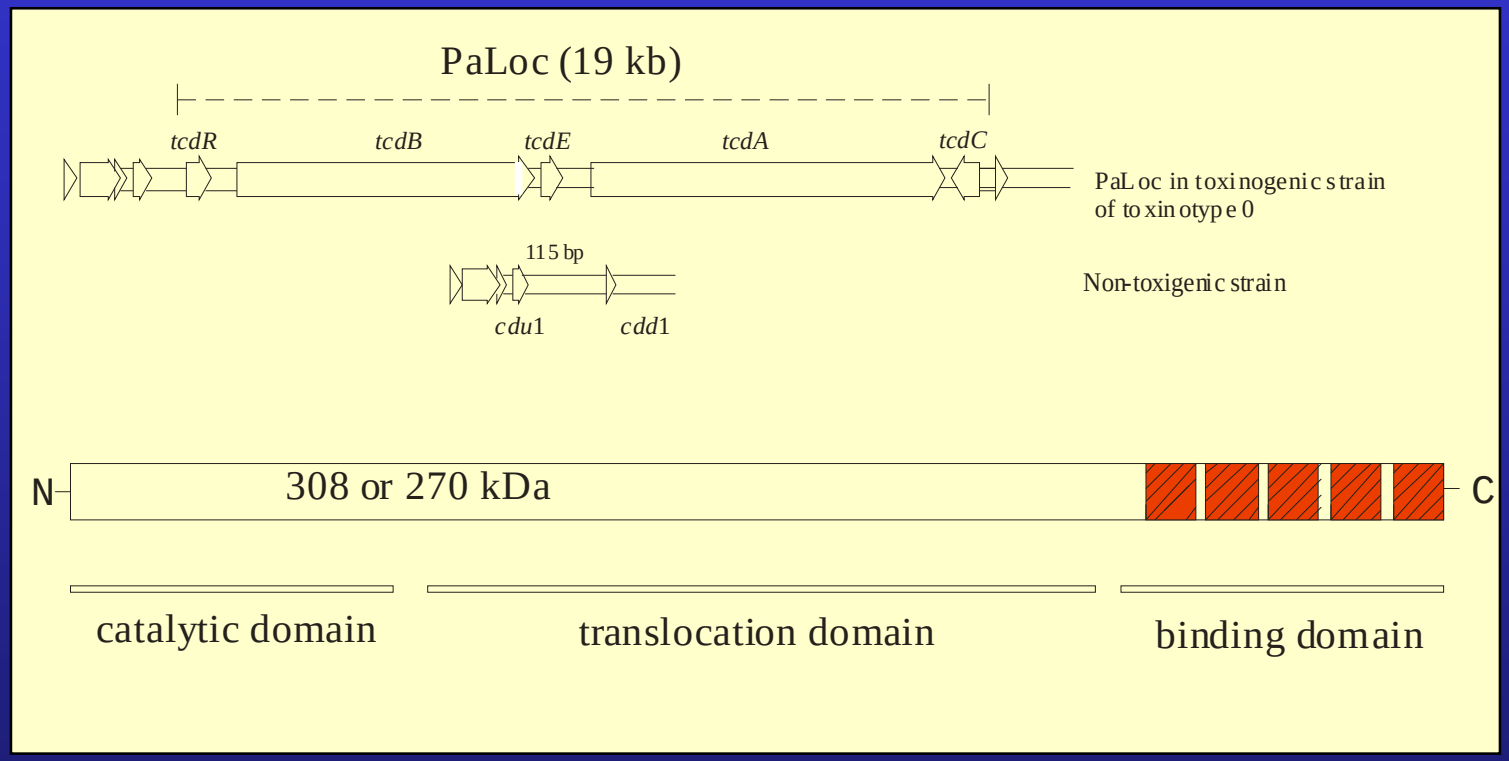
- Antibiotic resistance determinants

- Detection of toxin genes
  toxins A and B
  binary toxin

C. difficile toxin production types

	TcdB	TcdA	CDT	
Type 1	+	+	-	most prevalent
Type 2	+	-	-	0.2 - 12 %
Type 3	+	+	+	1.6 – 8 %
Type 4	+	-	+	very rare
Type 5	-	-	+	1.6 %
Type 6	-	-	-	20 %

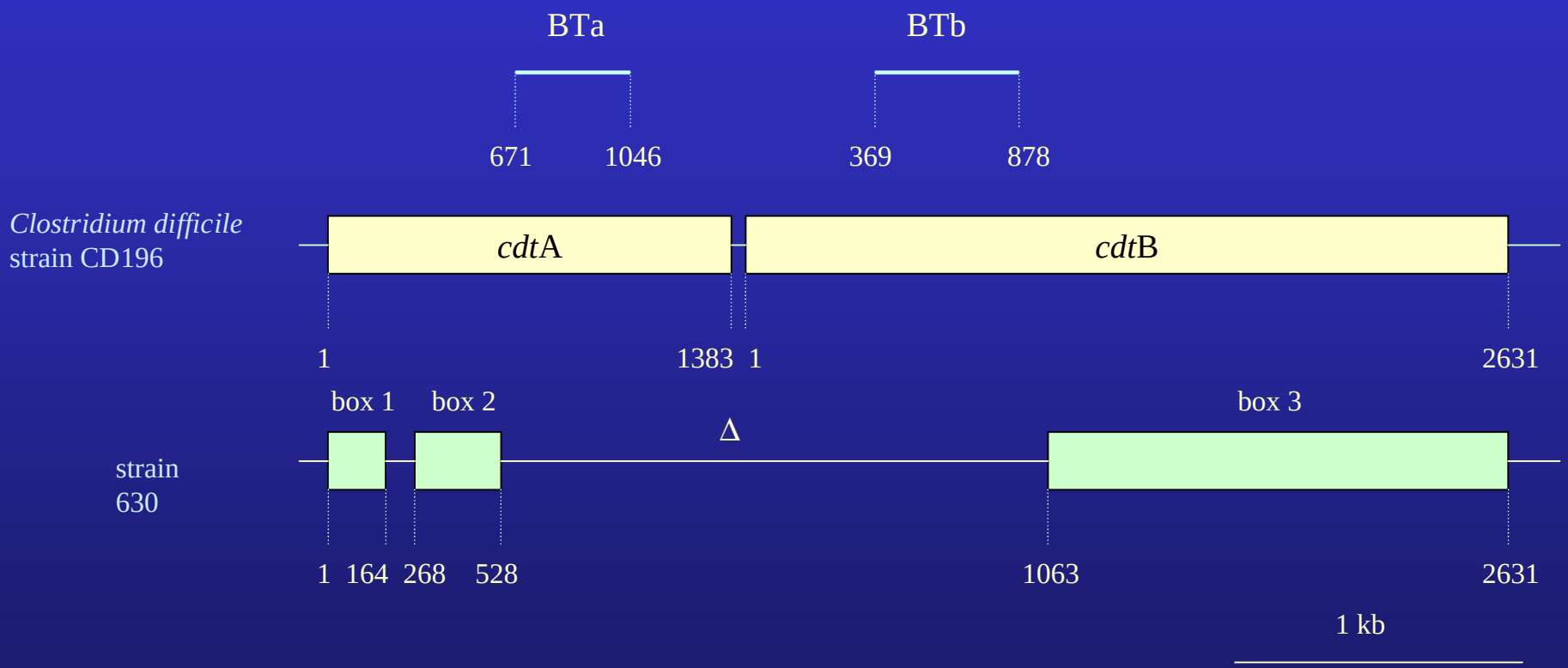
C. difficile toxins A and B – molecular detection



primers for tcdA and tcdB Kato et al., 1998; Rupnik et al.,1998

primers for 'noncytotoxic' strains Lok1/Lok3 Braun et al.,1996

C. difficile binary toxin genes



primers for binary toxin gene detection Stubbs et al., 2000

Why detecting binary toxin genes?

- Prevalence of binary toxin producing strains is increasing
  (Spigagalia and Mastrantonio, JMM, 2004)

time interval	before 1990	1991-1999	2000-2001
% of CDT+ strains	0	24	45

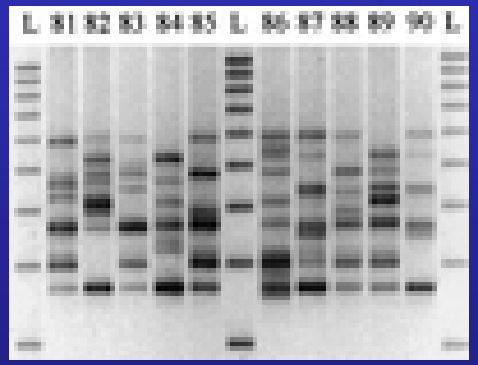
- Binary toxin positive strains more likely associated with severe disease
  (Barbut t al., JMM, 2005; Terhes et al., JCM, 2004)

C. difficile typing methods

- Serotyping
  - 30 serogroups
- PCR ribotyping
- PFGE
- REA
- PCR-RFLP (toxinotyping, *fliC*, *slpA*)
- Sequence based (MLST, MLVA – repeats; single locus)
- Microarray

Molecular epidemiology – mostly used methods

Ribotyping (Europe)

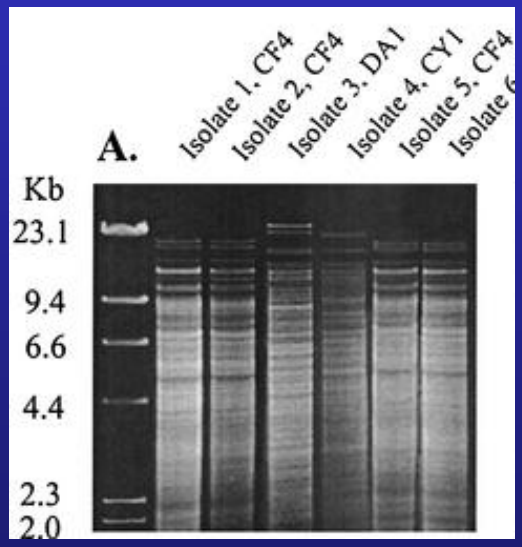


PCR of 16S-23S rDNA intergenic spacer region
160 ribotypes

Stubbs, JCM 1999

Bidet, JCM 2000

REA (USA)

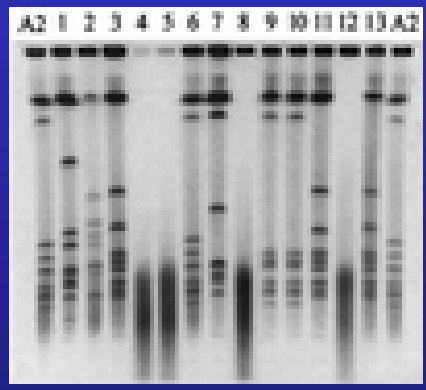


*Hin*dIII restriction of whole DNA

>100 REA groups
(Rea Types)

Gerding D., Chicago, USA

PFGE (North America)



*Sma*I restriction of whole DNA

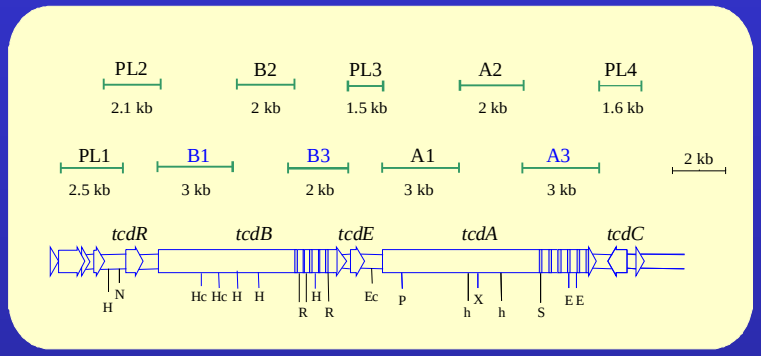
no large international collection

C. difficile typing methods - comparison

PCR Type	Serogroup	REA Group	REA Type	*tcdA* Deletion
17 (n=20)	F (n=16)	CF	CF1 (n=8)	1.8 Kb
			CF4 (n=4)	1.8 Kb
			CF2, CF3, CF5, CF6	1.8 Kb
	X (n=4)	CG	CG1 (n=3)	1.8 Kb
			CG3	1.8 Kb
47	F	CF	CF4	1.8 Kb
110	X	DA	DA1	None
36	A	CY	CY1	6.0 Kb

Johnson et al., JCM, 2003

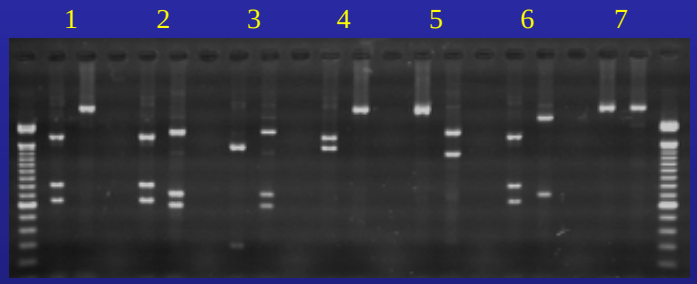
Toxinotyping of *C. difficile* strains



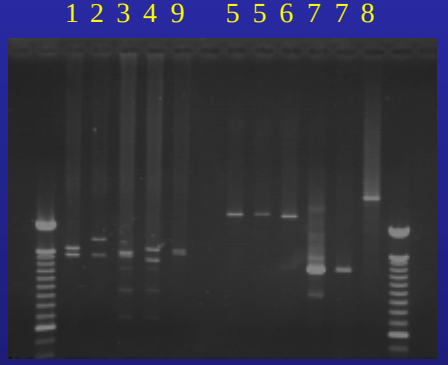
PCR method for screening changes in PaLoc

markers for toxinotyping

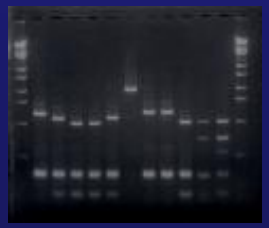
B1 PCR fragment



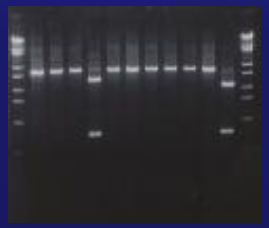
A3 PCR fragment



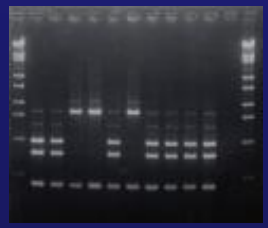
B2 PCR



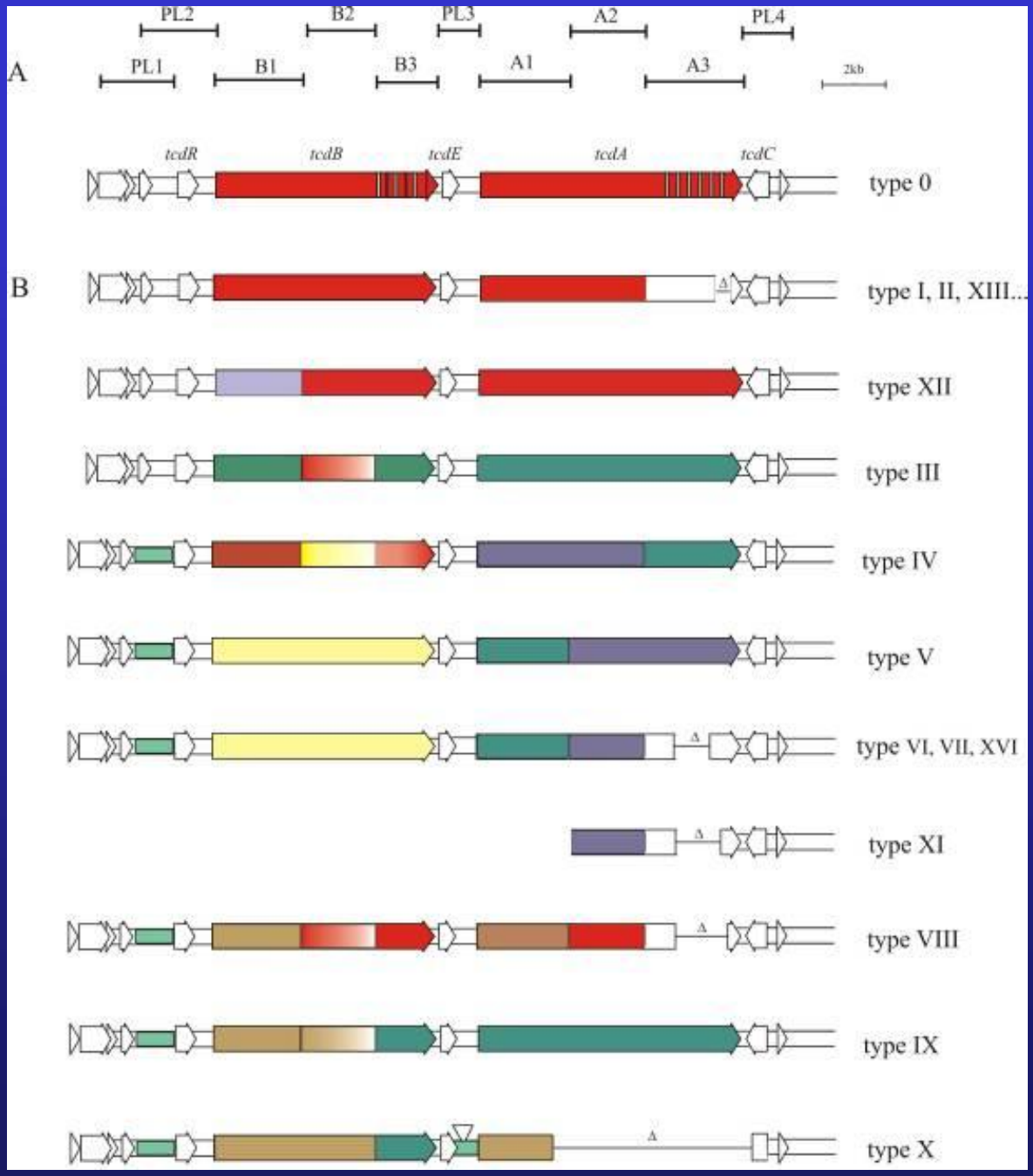
A1 PCR



A2 PCR



RFLPs in *tcd*B and *tcd*A


A
PL2
B2
PL3
A2
PL4
PL1
B1
B3
A1
A3
2kb
tcdR
tcdB
tcdE
tcdA
tcdC
type 0
B
type I, II, XIII...
type XII
type III
type IV
type V
type VI, VII, XVI
type XI
type VIII
type IX
type X

Variant strains - markers

	Reference strain VPI 10463	Minor modifications in PaLoc	Major modifications in PaLoc	Major modifications in PaLoc	
Toxinotypes	0	I, II, XII, XIII, XVIII, XIX, XX	XXI VIII (A-B+)	III, IV, V, VI, VII, IX, XI, XIV, XV, XXII, XXIII X, XVI, XVII (A-B+)	correlation with molecular typing methods
Binary toxin	negative	negative	negative	positive	

C. difficile typing

- Local environment
  - do I have identical strains
  - do I have prevalent type in hospital/ward
  - do I have hypervirulent type
- Broader geographic and time intervals
  - how related are strains from two/more geographic locations
  - is the hypervirulent type BI/NAP1/027 new

Typing in local environment

- comparison of isolated strains

  ribotyping, PFGE

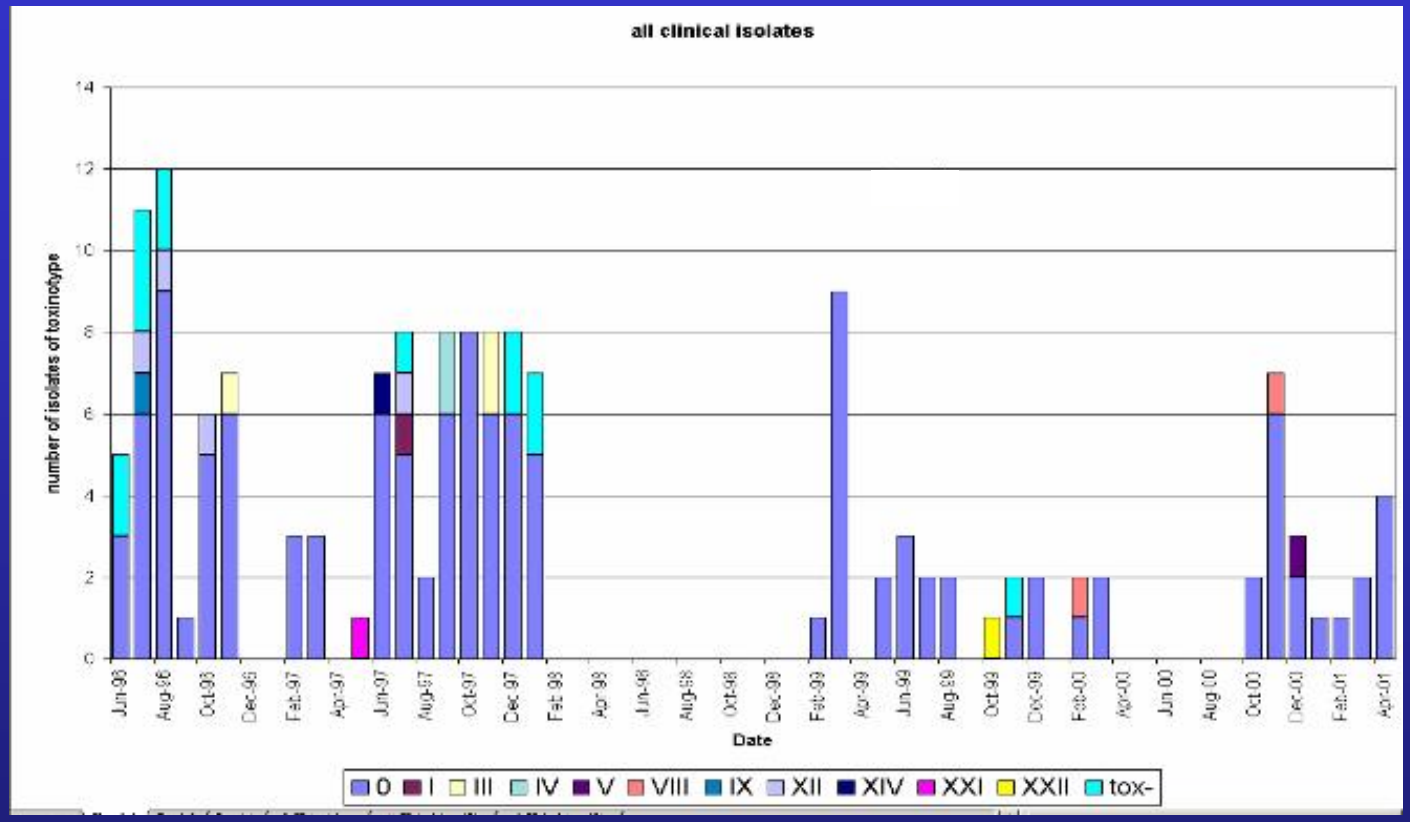
- detection of type BI/NAP1/027

  ribotyping, PFGE (comparison with reference strain)
  resistance pattern
  binary toxin gene positive
  toxinotype III
  tcdC (nonsense mutation at 117 – sequencing)

Variant strains – a hospital level

- Spain 01/2001 to 06/2001 9.5% variant strains
  5% A-B+
  4.5 % CDT+ (2 ribotypes only)
- Poland random selected from 1999-2001 isolates
  two variant types
  VIII and IV (8,6 % of tested strains)
- Japan 6 different hospitals 3 – 27% variant strains
  (1996 to 2000)
- USA 04/2001 to 03/2002 65,3% of CDT+ strains

Variant strains in a single hospital



250 bed tertiary University affiliated hospital , Chicago, USA
May 1996 to April 2001 153 *C. difficile* strains 11% variant strains
5,8% binary toxin positive

Geric, et al., J.Med.Micro., 2004

C. difficile – epidemiology of most prevalent types

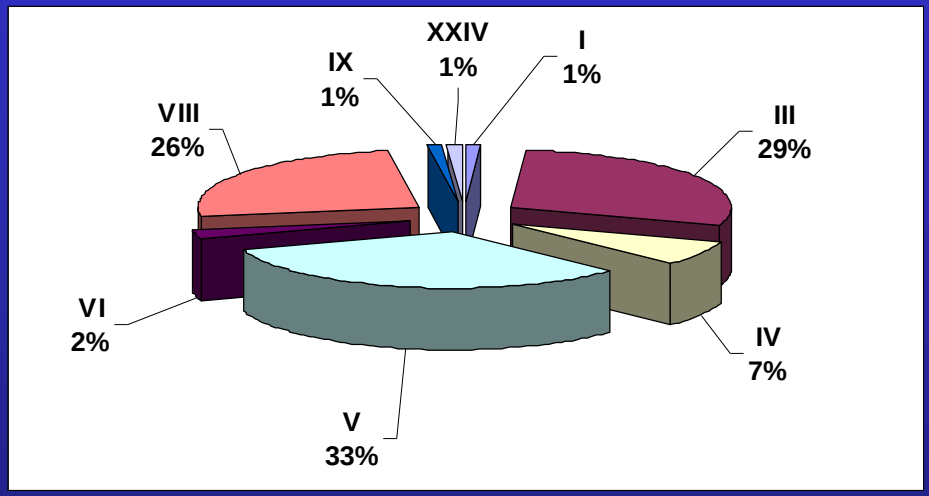
- current situaton in Europe
- A-B+ strains
- BI/NAP1/027 type
- comparison of human and animal strains

Distribution of variant strains in EU (2005)

- Barbut, Delmee, et al. (ESCMID Study group on *C. difficile)*
- 14 countries, 38 hospitals
- 2 months period in 2005
- 486 isolates
- 85,2 % toxinogenic (LCT+)

  25,6% variant strains

  17,2 % of toxinogenic strains were CDT+



- Ribotyping

  only 322 toxigenic strains of *C. difficile* were available / 66 different ribotypes found

  12 PCR ribotypes (001, 002, 012, 014, 017, 020, 027, 048, 077, 078, 126, 168) accounted for 65.5% of the strains.

Barbut et al, 2007 CMI submitted

ESGCD Study 2005 – ribotypes in EU

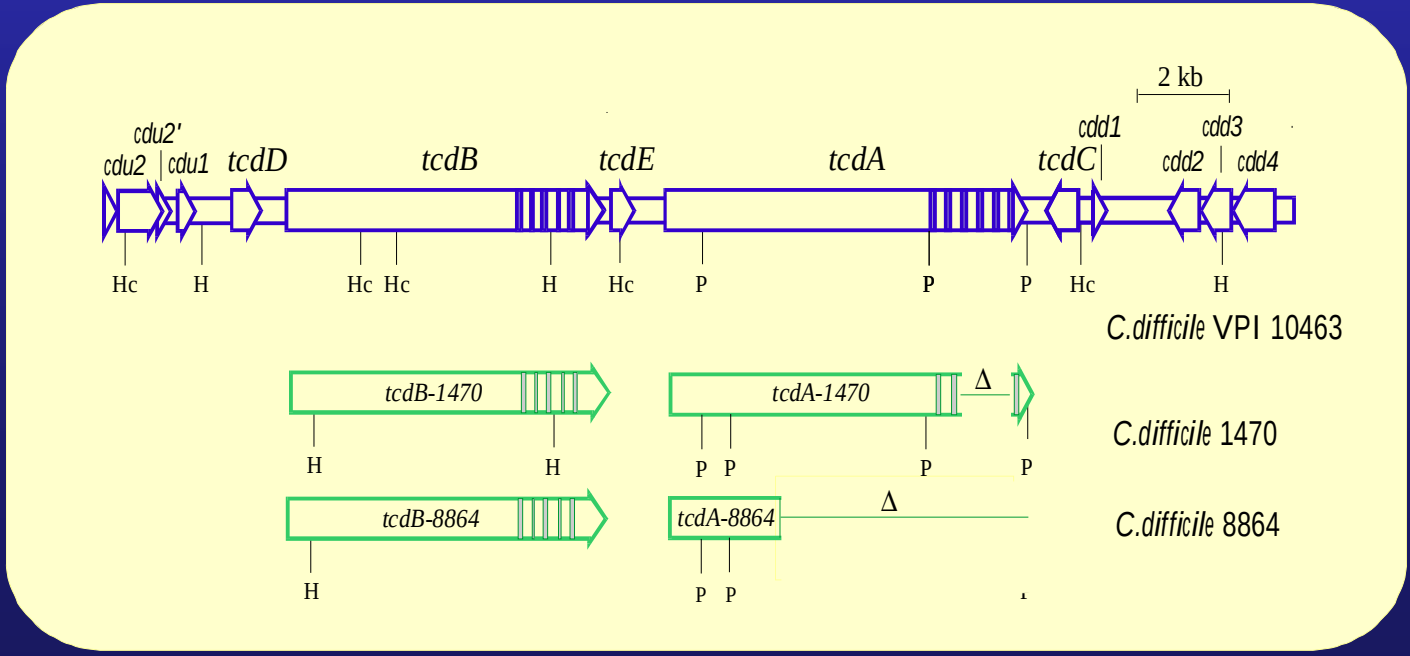
Country	No. of strains available for PCR ribotyping	No. of different PCR ribotypes	Major (>10%) PCR ribotypes (%)
Belgium	35	19	027 (31.4%)
France	33	13	014 (21.2%) 126 (15.2%) 002 (12.1%)
Germany	42	17	168 (21.4%) 001 (11.9%)
Great-Britain	8	4	077 (37.5%) 001 (25%) 014 (25%)
Greece	11	8	078 (27.3%) 017 (18.2%)
Hungary	42	18	014 (11.9%) 048 (11.9%)) 077 (11.9%)
Ireland	22	14	017 (18.2%) 156 (18.2%) 001 (13.6%)
Italy	19	12	020 (26.3%) 002 (10.5%) 023 (10.5%) 070 (10.5%)
Netherlands	20	9	027 (40%) 014 (20%)
Poland	16	8	017 (56.3%)
Spain	37	4	001 (73%) 020 (21.6%)
Sweden	16	12	017 (18.8%) 095 (12.5%) 023 (12.5%)
Switzerland	14	8	014 (21.4%) 054 (14.3%)
Turkey	7	6	001 (28.6%)

A-B+ *C. difficile* strains

- no production of TcdA (and CDT- or +)
- virulent
- diarrhea, PMC, outbreaks
- not detected with TcdA specific commercial diagnostic kits
- different types of A-B+ strains

First variant *C. difficile* strains

- TcdB-positive, TcdA-negative
- two groups
  - strain 8864
  - strains from serogroup F and X



Types of A-B+ *C. difficile* strains

toxinotype	characteristics	molecular basis for TcdA non-production	number of strains
VIII	1.8 deletion in *tcd*A	stop codon at aa position 47	>100
X	6 kb deletion in *tcd*A	rearrangement in PaLoc	1
XVI	similar to toxinotype V (A+B+)	not known	1
XVII	similar to toxinotype X	not known	1
0-like	identical to VPI 10463	not known	1
V-like	identical to toxinotype V	not known	1

Toxinotype VIII, serogroup F and X

- most prevalent A-B+ type
- first published description in 1993
  asymptomatic neonates
  not virulent in hamsters

- but
  serious (PMC) or lethal infections
  outbreaks
  (Canada, Netherlands, Ireland, Poland, Japan…)

Epidemic type BI/NAP1/027

- European *C. difficile* collection (ARU; UK) ribotyped

  2005 ribotype 027 <5 isolates
  2006 450 isolates

- Canadian *C. difficile* collection (2000-2005)

  Quebec none in 2000 to 2001
  75,2% in 2003 to 2004

  Alberta stable rates 7,4% of hospital associated strains

- American *C. difficile* collection (Chicago, USA) REA typed

  type BI present but rare

Epidemic type – emergence of antibiotic resistance

	type BI/NAP1/027		
	after 2001	before 2001	non-BI/NAP1/027
klindamicin	79	71	79
levofloksacin	100	100	96
gatifloksacin	100	0	42
moksifloksacin	100	0	42

(McDonald et al., NEJM, 2005)

Hypervirulent lineage in *C. difficile*?

- MLST (Lemee et al. 2005)

strains from different hosts, goegraphic sources, A+B+, A-B+, A-B-
strains from PMC and sever cases did not group together

- Whole Genome DNA Microarray analysis

all 027 strains related
all A-B+ strains related

C. difficile and animals

- *C. difficile* in healthy/diseased animals

  described in >10 animal species (camels, seals, deer, hamster…)
  cats, dogs, horses, piglets, calves

- animals as potential reservoir

  overlap of the types in humans and animals

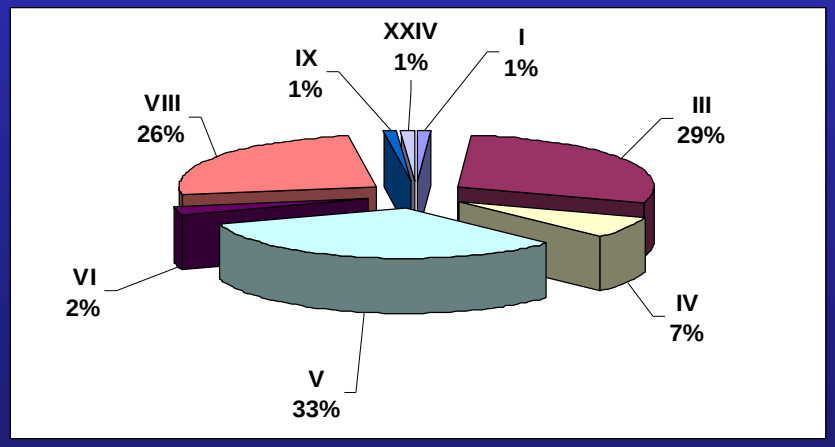
C. difficile types in humans and animals

- cats and dogs, humans (Australia) (O'Neill et al., Epidemiol. Infect. 1993)
  - no overlap
- horses, dogs, humans (Canada) (Arroyo et al., JMM 2005)
  - app. 5 ribotypes per species
  - 1 ribotype in all species (50% of all studied strains)
- calves (Canada) (Rodruigez-Palacios et al., Emerg.Infect.Dis., 2006)
  - 8 ribotypes
  - 7 of them also in human isolates (same time/geogr. area)
  - 078 (V), 017 (VIII), 027 (III), 033 (XI), 077 (0), 014 (0)

Toxinotypes in human and animal isolates (current snapshot)

Human isolates

EU study 2005 – hospital strains
non-variant 75 %
variant 25%



USA – community-associated strains
non-variant 52 %
variant strains of 9 toxinotypes
most prevalent III and V

Animal isolates

from 40 to 100 % variant strains

toxinotypes V, III, VIII, XI

horses
piglets
calves

EU and North America

Summary

- molecular methods can be used for direct detection or strain characterization
- follow the baseline in local environment
- laboratory is able to provide culture for typing
  severe cases
  outbreaks