



Institute of Public  
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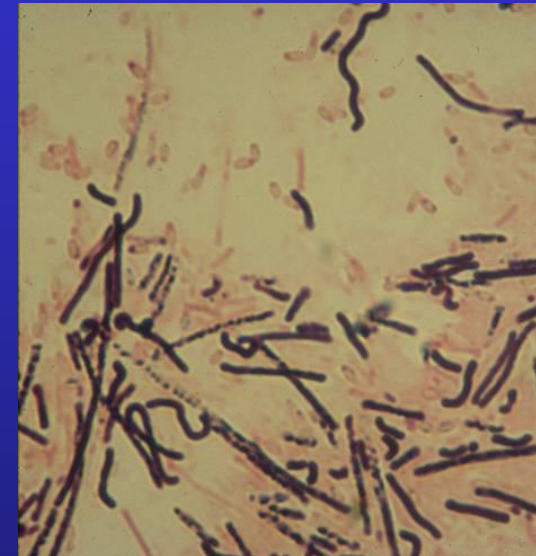
# *Clostridium difficile*: pathogenesis, diagnosis and treatment

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# *Clostridium difficile*

- anaerobic, sporogenic, Gram positive bacterium
- nosocomial infections
  - intestinal infections  
(diarrhoea, pseudomembranous colitis)
  - disturbed normal intestinal flora
  - extraintestinal infections
- nontoxigenic strains  
toxicogenic strains



Large Clostridial Toxins, LCT

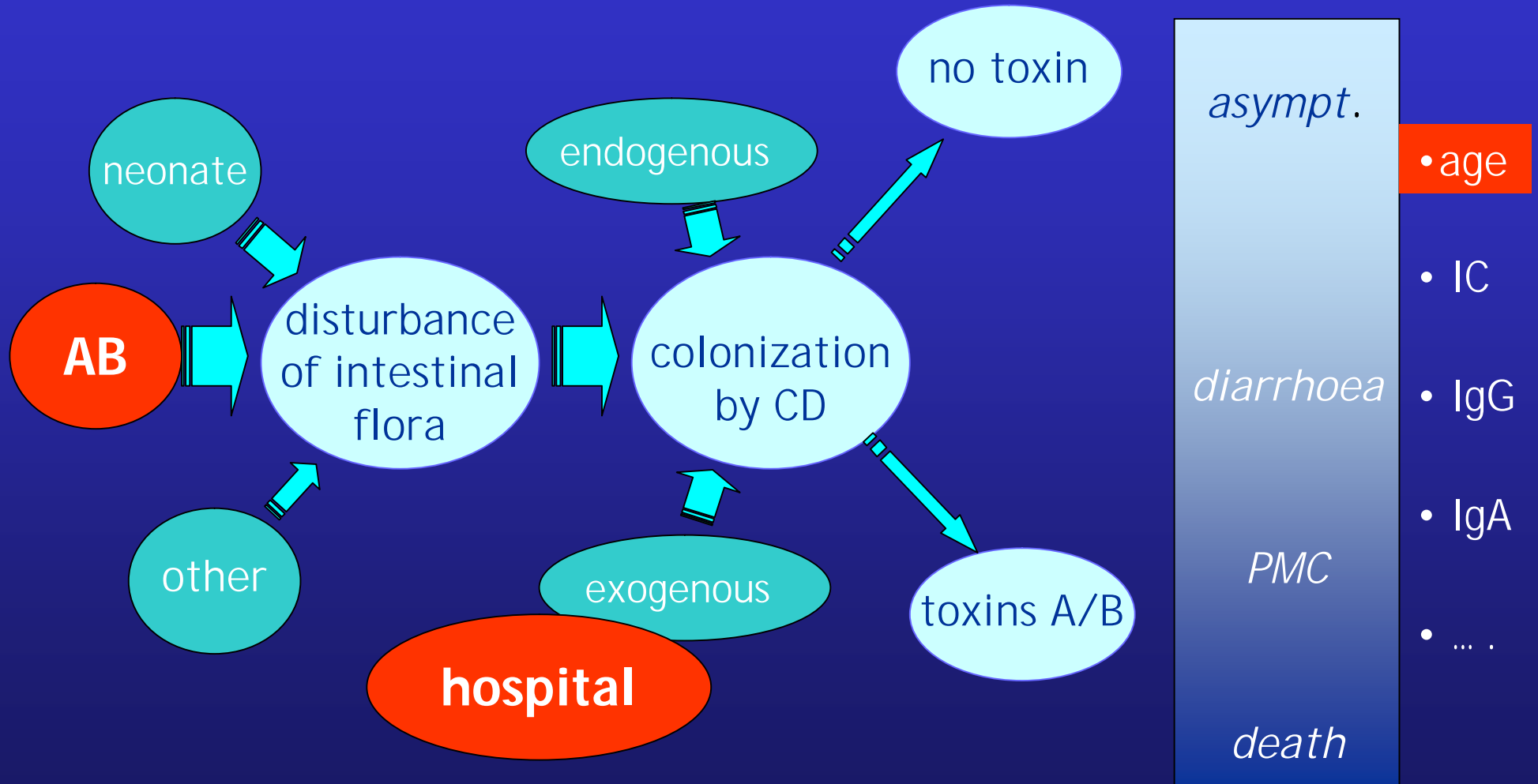
TcdA (enterotoxin)

TcdB (cytotoxin)

Binary toxin

CDT

# Risk factors for CDAD



# Pathogenesis

- Entry into host
- Colonisation
- Evasion of immune response
- Damage to the host
- Release and spread

# Pathogenesis

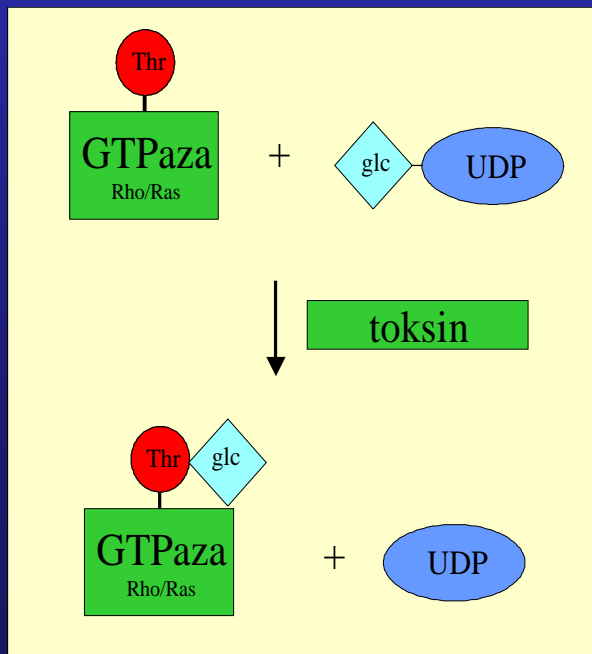
- **Entry into host**  
hospital environment, HCW – hands  
endogenous (source: previous hospitalization, animal contact, food?)  
5 to 30% asymptomatic carriers
- **Colonisation**  
disturbed gut flora  
adherence : SLP (surface layer proteins), flagellar proteins  
cultured cell experiments: strains differ in adherence
- **Release and spread**  
diarrhea  
spores!

# Large clostridial toxins (LCT)

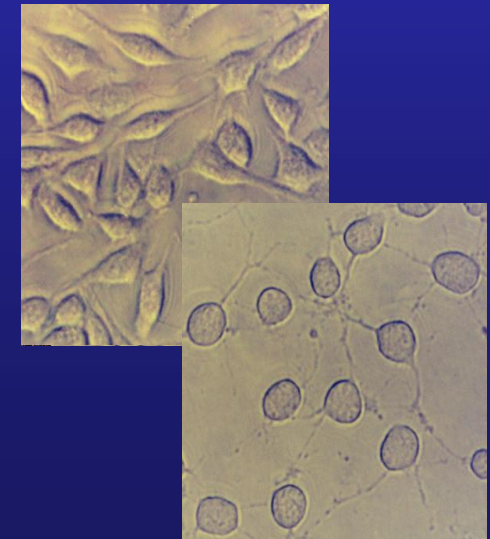
*C. difficile* (TcdA, TcdB)

*C. sordellii* (TcsH, TcsL)

*C. novyi* (Tcn $\alpha$ )



- size (250-300 kDa)
- cytotoxicity
- glycosyltransferases



# *C. difficile* binary toxin CDT

Large clostridial cytotoxins  
(LCT)

TcdA, TcdB

308 kDal  
270 kDal



glycosyltransferases

small GTPases → actin

cytotoxic

Clostridial binary toxins

CDT

binary structure

catalytic component  
50 kDal

binding component  
100 kDal



ADP-ribosyltransferases

actin

cytotoxic (trypsin activation)

# Binary toxin - additional virulence factor

- A- B- CDT+ strains from symptomatic patients
- related toxins (*C. perfringens* type E, *C. spiroforme*)  
only known virulence factors
- animal experiments – CDT has virulence potential (Geric et al., 2006)
  - hamster model      - - -
  - ligated loop assay    +++
- Binary toxin positive strains (A+B+CDT+ ) more likely associated with severe disease  
(Barbut t al., JMM, 2005; Terhes et al., JCM, 2004)

# Role of toxins A and B in the pathogenesis

- TcdA and TcdB main virulence factor
- Intestinal symptoms

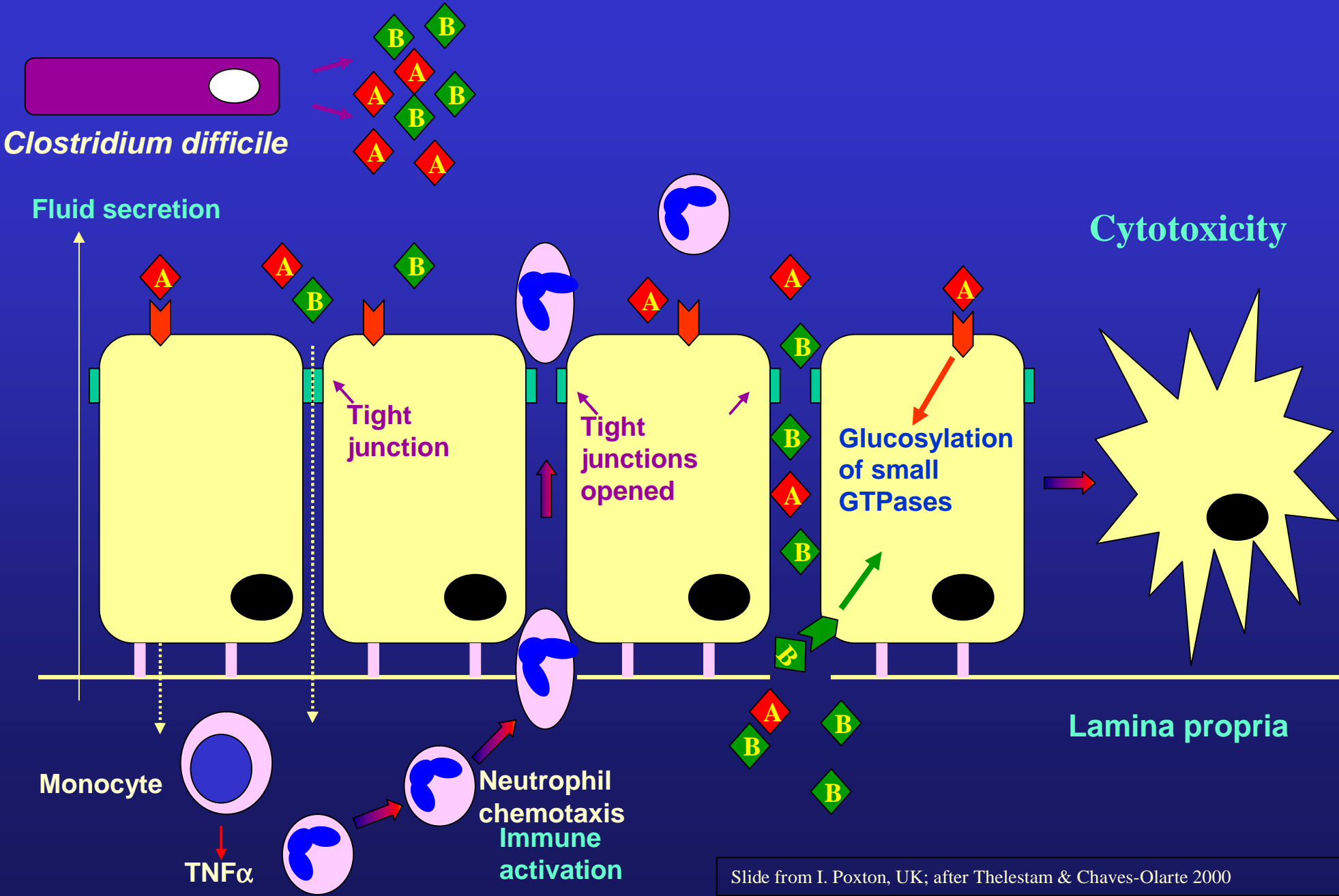
Cytotoxicity

Effects on immune system

Effects on the intestinal innervation (via immune system?)

- Systemic action?

# Actions of *C. difficile* Toxins A and B on intestinal epithelium



Slide from I. Poxton, UK; after Thelestam & Chaves-Olarte 2000

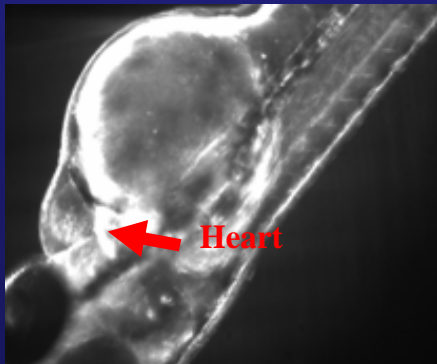
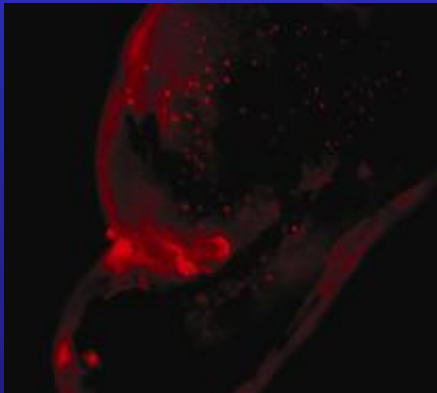


# Zebrafish Embryos as a Model to Study the Systemic Effects of TcdB

(Hamm EE, Voth DE, Ballard JD., PNAS, 2006)

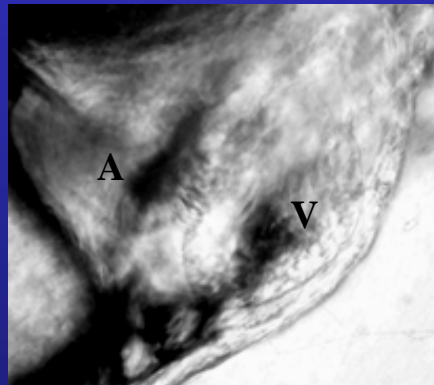
## Localization at pericardial region

TcdB<sup>Alexa546</sup>

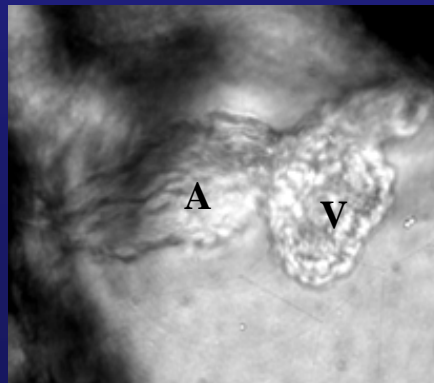


## Cardiac damage

Control



TcdB



## Edema in TcdB-Treated Embryos

Control



TcdB



## *C. difficile*: current clinical challenges

- CDAD rates are increasing and a common epidemic *C. difficile* strain has been found in the US, Canada, and Europe.
- More severe CDAD with higher mortality and higher rates of colectomy is being reported.
- Absence of a treatment that will prevent recurrence of CDAD: Recurrence rates are 20-35% and appear to be increasing.
- The effectiveness of metronidazole for treatment of CDAD is being questioned.

# Treatment

- Antibiotics

metronidazole or vancomycin

- are antimicrobials that disrupt the normal flora; susceptible to relapse (same organism) or reinfection (new organism)
- lower clinical response to metronidazole
- resistance

- Very narrow spectrum antimicrobials

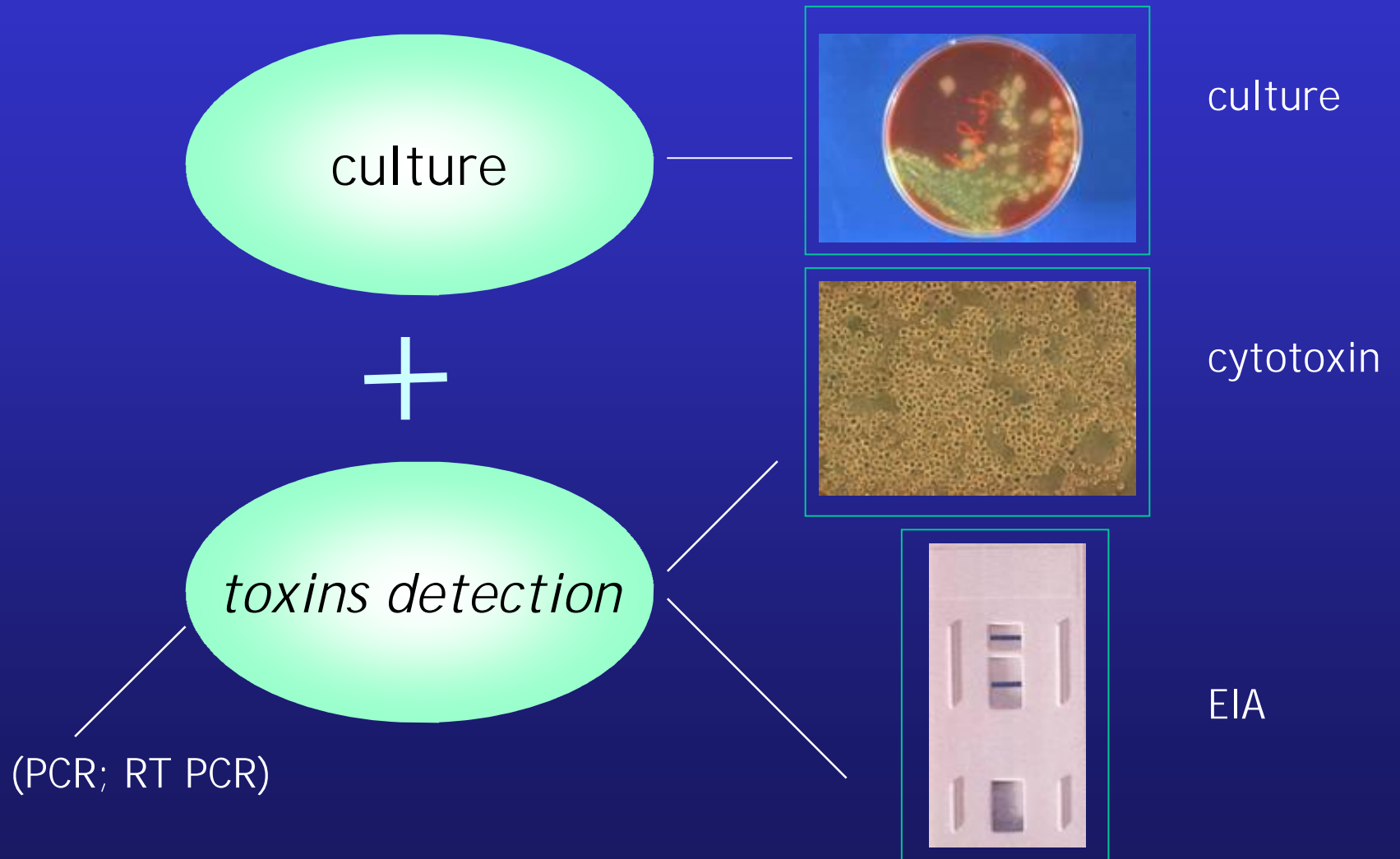
- Toxin binding agents

- Probiotics

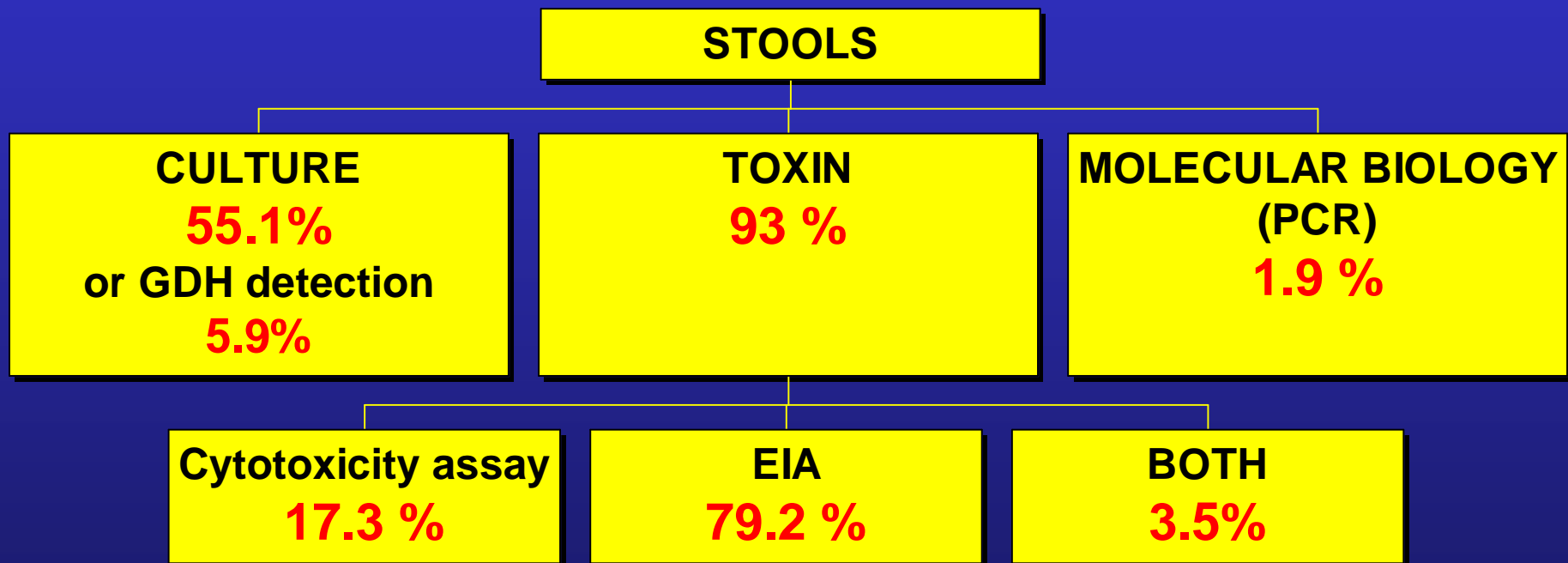
- Faecal transplants

- Vaccines

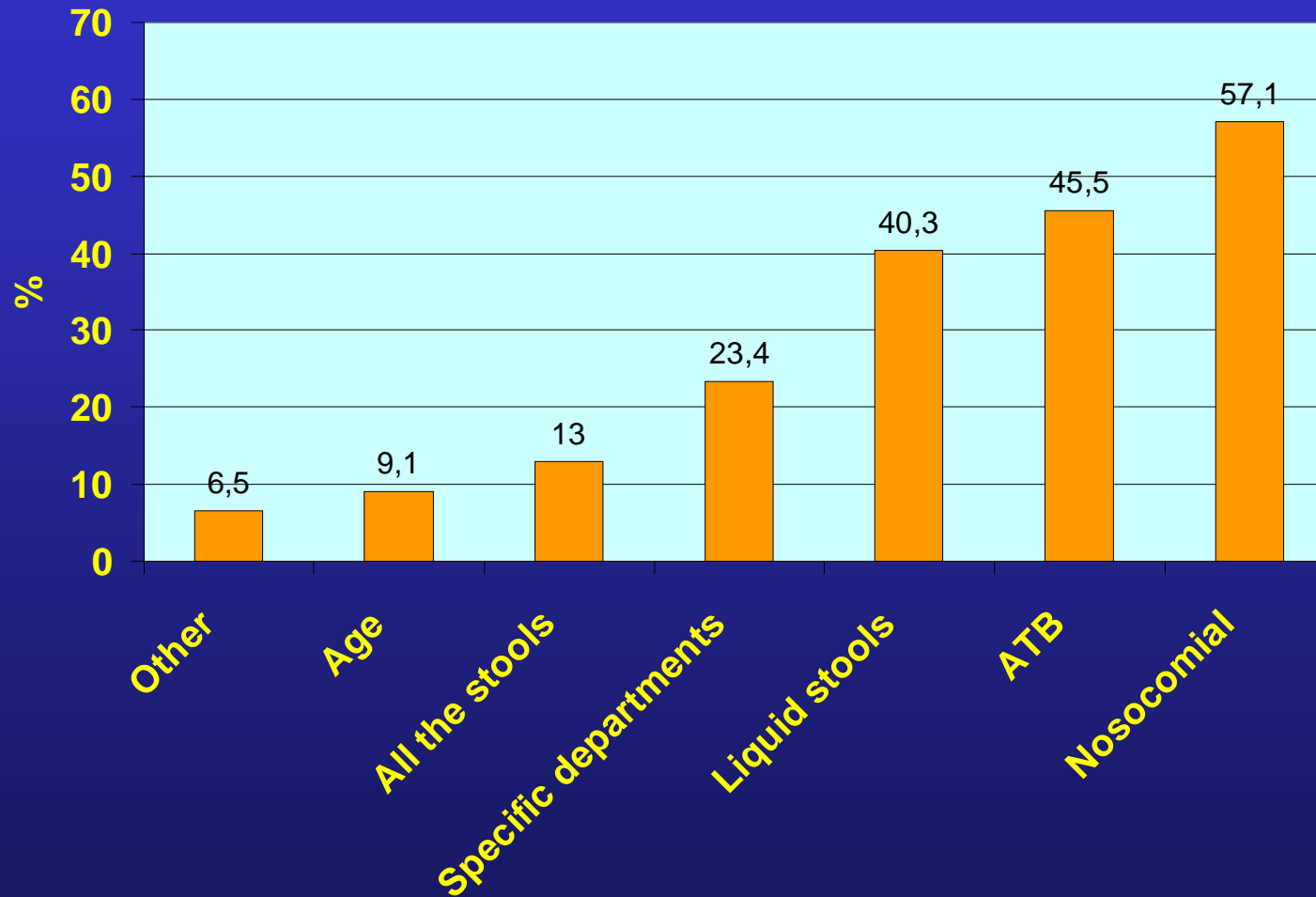
# Diagnosis of *C. difficile*



# EU survey for diagnostic methods for *C. difficile*



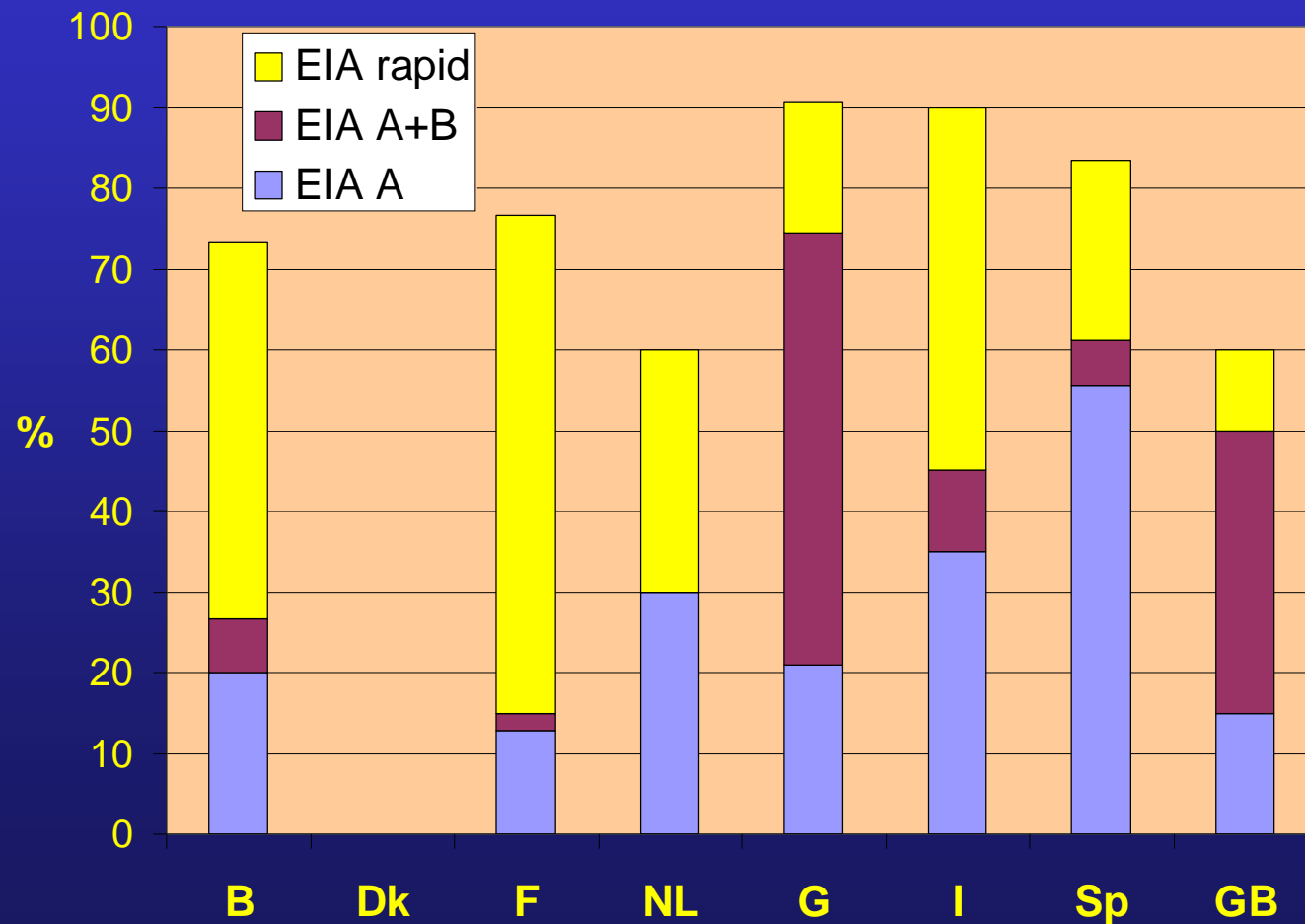
# Criteria for *C. difficile* testing



# EU study : culture

- **Media :** home made : 28.4%
  - » commercial (CCA) : 68.6% (Biomérieux>>Oxoid, Becton)
- **Inoculation** (no difference between countries) :
  - direct : 68.6%
  - enrichment step : 26.5%
  - both : 4.9 %
- **Incubation**
  - chambers : 29.4 % (100% for Sp and Dk)
  - jars : 70.6% (Anoxomat : 29.2% but 100% for NL and 61 % for B)
- **Time incubation** : 48 h (82.4%) (no difference between countries)

# EU study : types of toxin tests used



## Which toxins to test

### *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 %           |

# Detecting binary toxin genes

## Why and how

- Prevalence of binary toxin producing strains is increasing  
(Spigaglia 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)
- Detection by molecular methods  
(Stubbs et al., FEMS Lett., 2000)

# *C. difficile* – antibiotic resistance

- Importance

- Therapeutic antibiotics (metronidazole, vancomycin)

- resistant strains rarely reported

- MIC are increasing

- clinical failure after metronidazole

- Other antibiotics

- effect on colonization after antibiotic therapy

- clindamycin, 3<sup>rd</sup> gen cephalosporins, fluoroquinolones

- Testing

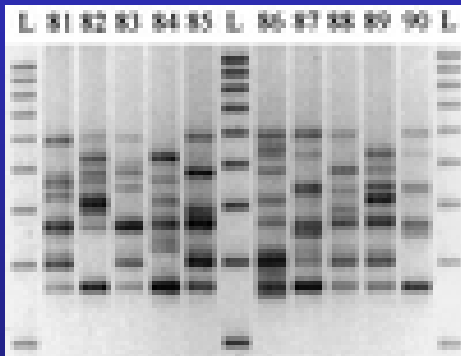
- Etest

## *C. difficile* typing methods

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

# Molecular epidemiology – mostly used methods

## Ribotyping (Europe)



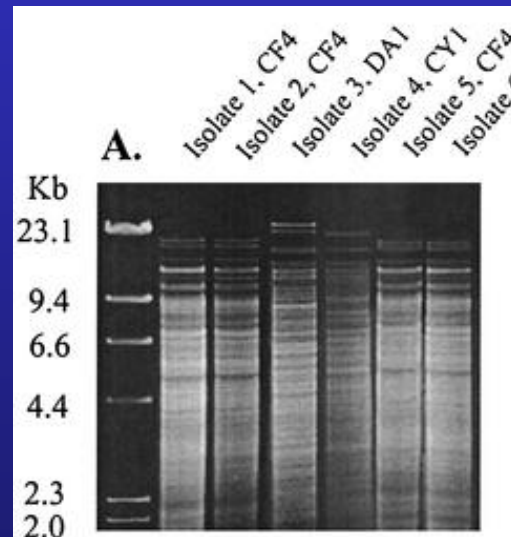
PCR of 16S-23S rDNA intergenic spacer region

160 ribotypes

Stubbs, JCM 1999

Bidet, JCM 2000

## REA (USA)

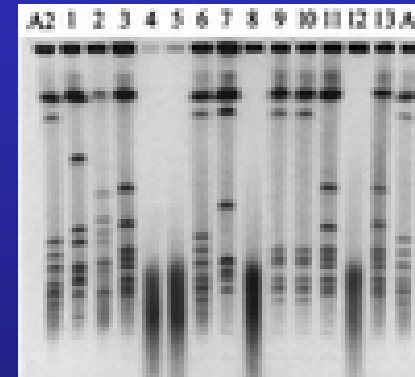


*Hind*III restriction of whole DNA

>100 REA groups  
(Rea Types)

Gerding D., Chicago, USA

## PFGE (North America)



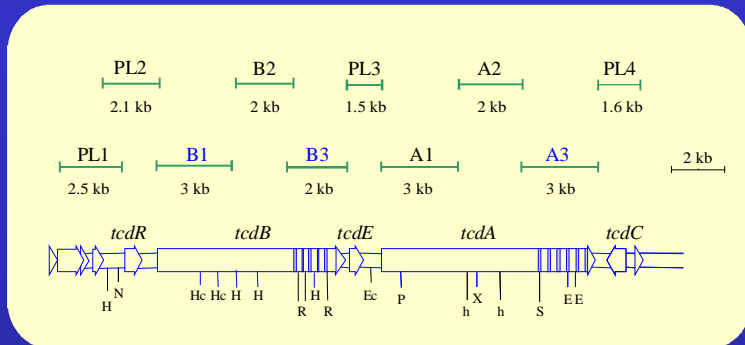
*Sma*I restriction of whole DNA

no large international collection

# *C. difficile* typing methods - comparison

| <u>PCR Type</u> | <u>Serogroup</u> | <u>REA Group</u> | <u>REA Type</u>    | <u>tcdA Deletion</u> |
|-----------------|------------------|------------------|--------------------|----------------------|
| 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               |

# Toxinotyping of *C. difficile* strains

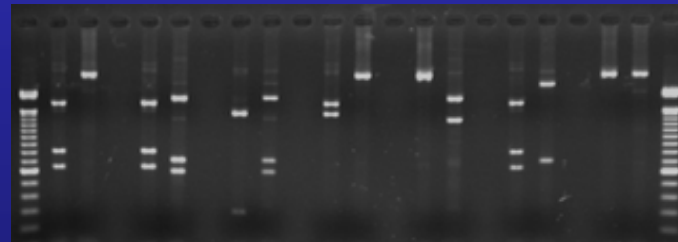


PCR method for screening changes in PaLoc

markers for toxinotyping

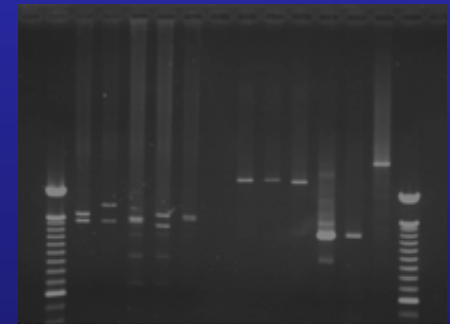
B1 PCR fragment

1 2 3 4 5 6 7



A3 PCR fragment

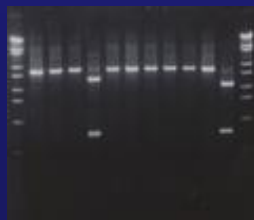
1 2 3 4 9 5 5 6 7 7 8



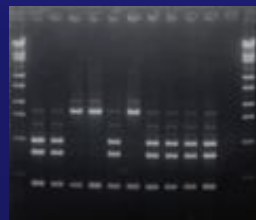
B2 PCR



A1 PCR



A2 PCR



RFLPs in *tcdB* and *tcdA*

# Variant strains - markers

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

# Typing in local environment

- comparison of isolated strains (prevalent type?, outbreak?)

ribotyping, PFGE

- detection of type BI/NAP1/027

ribotyping, PFGE (comparison with reference strain)

resistance pattern

binary toxin gene positive

toxintype III

tcdC (nonsense mutation at 117 – sequencing)

# 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+)

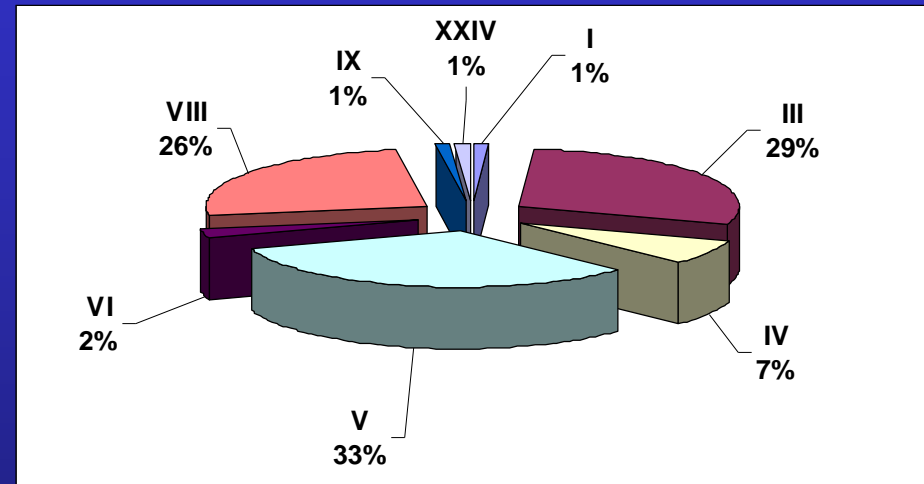
25,6% variant strains

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.



# 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%)<br>126 (15.2%)<br>002 (12.1%)                |
| Germany       | 42  | 17                             | 168 (21.4%)<br>001 (11.9%)                               |
| Great-Britain | 8   | 4                              | 077 (37.5%)<br>001 (25%)<br>014 (25%)                    |
| Greece        | 11  | 8                              | 078 (27.3%)<br>017 (18.2%)                               |
| Hungary       | 42  | 18                             | 014 (11.9%)<br>048 (11.9%)<br>077 (11.9%)                |
| Ireland       | 22  | 14                             | 017 (18.2%)<br>156 (18.2%)<br>001 (13.6%)                |
| Italy         | 19  | 12                             | 020 (26.3%)<br>002 (10.5%)<br>023 (10.5%)<br>070 (10.5%) |
| Netherlands   | 20  | 9                              | 027 (40%)<br>014 (20%)                                   |
| Poland        | 16  | 8                              | 017 (56.3%)  |
| Spain         | 37  | 4                              | 001 (73%)<br>020 (21.6%)                                 |
| Sweden        | 16  | 12                             | 017 (18.8%)<br>095 (12.5%)<br>023 (12.5%)                |
| Switzerland   | 14  | 8                              | 014 (21.4%)<br>054 (14.3%)                               |
| Turkey        | 7   | 6                              | 001 (28.6%)  |

# *C. difficile* : emerging issues

- Spread of highly virulent clones

A-B+ strains (ribotype 017)

new epidemic type BI/NAP1/027

- *C. difficile* in animals

## 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

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

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

# Emergence of new highly virulent *C. difficile* type in Canada

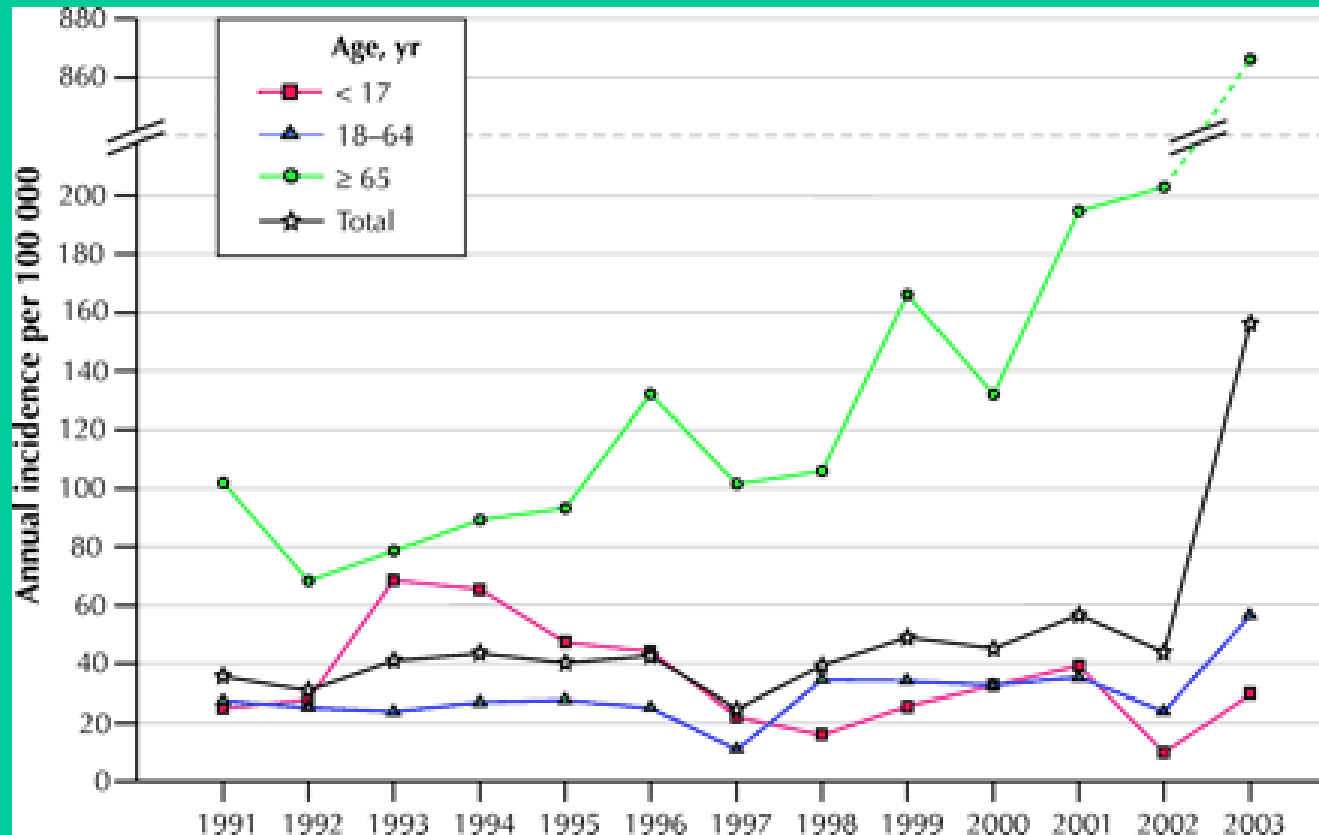


Fig. 1: Annual incidence (per 100 000 population) of *Clostridium difficile*-associated diarrhea (CDAD) in Sherbrooke, Que., 1991–2003.

Pepin et al., JAMC, 2004

# Characteristics of new *C. difficile* – BI/NAP1/027

- associated with hospital outbreaks
- increased disease severity
- increased mortality

- Typing

toxintyping (mutations in PaLoc)

REA (restriction fragments of genomic DNA)

PFGE

ribotyping

toxintype III

type BI

type NAP1

type 027

- variant toxins TcdA in TcdB
- higher in vitro toxin production (TcdA in TcdB)
- binary toxin
- deletion in regulatory gene *tcdC*
- antibiotic resistance (fluoroquinolones)

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

(McDonald et al., NEJM, 2005)

# *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

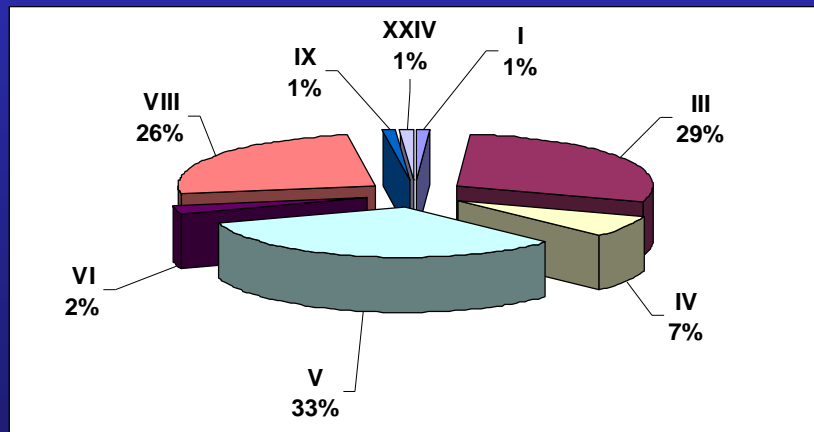
- cats and dogs, humans (Australia) (O'Neill et al., Epidemiol. Infect. 1993)  
no overlap
- horses, dogs, humans (Canada) (Arroyo et al., JMM 2005)  
app. 5 ribotypes per species  
1 ribotype in all species (50% of all studied strains)
- calves (Canada) (Rodruiguez-Palacios et al., Emerg.Infect.Dis., 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

## 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

- think of *C. difficile* in any symptomatic patient with no other known pathogen (regardless of age, antibiotic exposure, community-associated)
- follow the hospital baseline
- laboratory is able to provide culture  
severe cases  
outbreaks