Antibiotic resistance in *Clostridium difficile* – recent advances

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New traits of antibiotic resistance in *C. difficile* clinical isolates:

- resistance to multiple antibiotics
- reduced susceptibility to antibiotics used for treatment
- rapid spreading and persistence of resistance
Antibiotics and CDI

Antibiotic treatment is the main risk for CDI

*C. difficile* infection (CDI) occurs when the intestinal microflora is altered or disrupted, allowing *C. difficile* to colonize the gut and produce toxins

Resistance to a wide range of antibiotics allow *C. difficile* to colonize and infect in the presence of drugs

- The antibiotics more frequently implicated in causing CDI include:
  - **Cephalosporins** (intrinsic)
  - **Clindamycin** (historical)
  - **Fluoroquinolones** (recent - associated to emergence and spread of RT027)
### Phenotypic traits of *C. difficile* clinical isolates

**Antimicrobial-Resistant Strains of *Clostridium difficile* from North America**

Fred C. Tenover, Isabella A. Tickle, and David H. Persing

<table>
<thead>
<tr>
<th>% of resistance</th>
<th>% of the most frequent RTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLI 41.50</td>
<td>027 49.00</td>
</tr>
<tr>
<td>MXF 38.00</td>
<td>002 10.4</td>
</tr>
<tr>
<td>RIF 7.90</td>
<td>106 10.4</td>
</tr>
<tr>
<td>MTZ 0.0</td>
<td>078 8.6</td>
</tr>
<tr>
<td></td>
<td>053 7.4</td>
</tr>
</tbody>
</table>

AAC 2012. 56: 2929 -2932

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**Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes**

J. Freeman¹, J. Vernon², K. Morris³, S. Nicholson⁴, S. Todhunter⁵, C. Longshaw⁶ and M. H. Wilcox⁷ and the Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent *Clostridium difficile* Ribotypes Study Group

<table>
<thead>
<tr>
<th>% of resistance</th>
<th>% of the most frequent RTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLI 49.62</td>
<td>027 12.5</td>
</tr>
<tr>
<td>MXF 39.99</td>
<td>001/072 9.0</td>
</tr>
<tr>
<td>RIF 13.40</td>
<td>014 8.1</td>
</tr>
<tr>
<td>IMP 7.41</td>
<td>078 8.1</td>
</tr>
<tr>
<td>CHL 3.70</td>
<td>020 4.2</td>
</tr>
<tr>
<td>MTZ 0.11</td>
<td></td>
</tr>
<tr>
<td>VAN 0.87</td>
<td></td>
</tr>
<tr>
<td>FDX 0.0</td>
<td></td>
</tr>
<tr>
<td>TIG 0.0</td>
<td>Clin Microbiol Infect 2015. 21:248.e9-248.e16</td>
</tr>
</tbody>
</table>

AAC 2012. 56: 2929 -2932
Resistance to CLI and MXF is a common trait of epidemic strains but...

Percentage of resistance

(based on 10 studies published between 2012 and 2014 - 486 C. difficile isolates)
High level of resistance to RIF has been observed in 17/22 EU countries. In particular:

% of resistant strains

- Czech Republic 63.64 (RT017, RT027, RT176, RT001/072)
- Italy 62.37 (RT018, RT356)
- Denmark 56.52 (RT027)
- Hungary 58.67 (RT027)

...an increasing percentage of resistance to RIF has been also observed in some RTs

Clin Microbiol Infect 2015. 21:248.e9-248.e16
C. difficile: a multi-drug resistant pathogen

- 55% of EU resistant strains were MDR
- 49% of MDR isolates were resistant to four different classes of antibiotics: ERY, CLI, MXF and RIF
- MDR strains belonged predominantly to: 001(39%), 017 (18%) and 012 (12%)
Review of 12 studies published between 2012 and 2014 (370 MDR *C. difficile* isolates)

- The most common resistance patterns include resistance to: CLI, FQs, CFs, ERY and RIF

- RTs most commonly associated to MDR: 001/072, 012, 017, 027, 046, 176, 018

- The percentage of MDR strains ranges between 13 and 100% depending from the hospitals and the geographic regions

5th International *Clostridium difficile* Symposium - 19th to 21° May 2015
Lower antibiotic resistance levels among countries with a greater diversity of *C. difficile* RTs (Norther and Western Europe).

It is probably due to mandatory reporting programmes with consequent decrease of endemic RTs rates. 

*Clin Microbiol Infect* 2014. 21:248.e9-248.e16

- In Italy: RT018 predominat from 2006 to 2013 when RT356 emerged. 

- RT018 and 356 show 87.5% of genetic similarity. 
  Both RTs are MDR (MXF, RIF, ERY)
  *Microbiologia Medica* 2014; 29:4722

- Selection for RIF resistance probably secondary to drug exposure (used in Italy for more than 20 years)

5th International *Clostridium difficile* Symposium - 19th to 21st May 2015
Reduced susceptibility to MTZ: an emerging phenotype

- Metronidazole (MTZ) is the drug of choice in the treatment of mild-to-moderate CDI

- Frequency of treatment failure or recurrence of CDI is high
  
  Treatment failure is higher with MTZ than VAN (22.4% vs 14.2%; P=0.002)
  
  Recurrence rate is similar (27.1% vs 24.0%, P=0.26)


- Recently, reduced susceptibility to MTZ and heteroresistance have raised in *C. difficile* population

  *JAC.* 2008. 62: 1046–52
  *AAC.* 1999. 43: 2607–11
  *JAC.* 2013. 68: 362–365
A higher geometric mean MICs to MTZ has been observed in some RTs

Epidemiological cut-off (ECOFF) for reduced susceptibility to MTZ is defined as MIC > 2mg/L (EUCAST)

<table>
<thead>
<tr>
<th>Source</th>
<th>MIC</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobe. 2015. 33:105-108</td>
<td>RT027</td>
<td>1.1 mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Canada)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associated with recurrent diseases</td>
</tr>
<tr>
<td>Clin Microbiol Infect 2014. 21:248.e9-248.e16</td>
<td>RT027</td>
<td>1.42 mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(EU and UK)</td>
</tr>
<tr>
<td></td>
<td>RT106</td>
<td>0.65 mg/L</td>
</tr>
<tr>
<td></td>
<td>RT001/072</td>
<td>0.65 mg/L</td>
</tr>
<tr>
<td></td>
<td>RT356</td>
<td>0.61 mg/L</td>
</tr>
<tr>
<td>J Antimicrob Chemother 2013. 68: 362-365</td>
<td>RT010</td>
<td>1.5 mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non toxigenic</td>
</tr>
</tbody>
</table>

MICs of others RTs range between 0.62 – 0.95 mg/L
C. difficile strains resistant to MTZ (MICs between 3 and ≥ 256 mg/L) have been reported (EUCAST-ECOFF > 2mg/L ; CLSI-MTZ breakpoint ≥ 32mg/L).

Review of 21 papers published between 1997 and 2014

**Human**
RT027; RT001; RT078; RT010

**Animal**
RT078 (swine, dog); RT010 (dog)

Horse
Swine
Dog
Zebra

**Environment**
RT010
Identification of strains with reduced susceptibility to MTZ is not easy...

Often, strains with reduced susceptibility to MTZ do not maintain the MIC values after passages in antibiotic free media or after freeze/thaw


Agar Incorporation Method (AIM) has been demonstrated the method of choice to detect colonies with reduced susceptibility to MTZ

Etest<ADM< AIM


... so these strains can be underestimated

*C. difficile* strains characterized as susceptible to MTZ have been associated with cases of treatment failure

Exposure to MTZ selects colonies of strains RT010 and 001 with increased MICs.

Antibiotic subinhibitory concentrations could have a relevant role in selecting and maintaining colonies with higher MICs.


Mean concentration of MTZ in the feces of patients during treatment ranges from < 0.25 to 9.5 mg/L.

It can be hypothesized that these concentrations can be insufficient for the treatment of CDI due to strains with higher MICs.

The majority of *C. difficile* strains are still susceptible to VAN and FDX

Epidemiological cut-off (ECOFF) for reduced susceptibility to VAN is defined as MIC > 2mg/L (EUCAST)

*C. difficile* isolates with MICs >1 mg/L for FDX have been defined intermediate

*Clin Microbiol Infect.* 2014. 21:248.e9-248.e16

- Vancomycin (VAN) is a first-line option in severe CDI
- Few strains with reduced susceptibility to VAN (MICs range 4-16 mg/L) as been found
  
  *Clin Microbiol Infect.* 2014. 21:248.e9-248.e16
  
  AAC. 2002. 46:1647-50
  AAC. 2007. 51: 2716-19
  AAC. 2012. 56: 3943-49
  
  *Iranian Red Crescent Medical Journal.* 2013. 15: 704-11
  
  *International Journal of Antimicrobial Agents* 41 (2013) 80–84

- Fidaxomicin (FDX) is currently used in case of severe CDI and recurrences
- *C. difficile* isolates are susceptible to FDX
  
  MIC<sub>50</sub> ≤0.016 - 0.25 mg/L
  
  MIC<sub>90</sub> 0.125 - 0.5 mg/L

  *Expert Rev Anti Infect Ther.* 2010. 8: 555-64
  
  *Clin Microbiol Infect.* 2014. 21:248.e9-248.e16
  
  2010. 8: 555-64; *Anaerobe.* 2003. 47.2334-38

- Only strain with MIC=16 mg/L have been identified

  *CID.*2012. 55 (Suppl 2): S143
C. difficile mutants with reduced susceptibility to VAN (MICs 4-16 mg/L) or FDX (MICs 1-4 mg/L) has been isolated in vitro after passages on medium containing antibiotics. Since the fecal concentration of these antibiotics is high (2000 µg/g for VAN and >1000 µg/g for FDX) the clinical significance of strains with reduced susceptibility is unclear.

Mutants show mutations mediating target modification (RNA polymerase, cell wall)
Emergence, spread and persistence of *C. difficile* antibiotic resistance: an increasingly complex topic
Mobile Genetic Elements (MGEs)

- Any region of nucleic acid that can move from one part of the genome to another or between genomes

- About 11% of the *C. difficile* genome is made up of MGEs

- MGEs provide *C. difficile* of remarkable genetic plasticity
C. difficile contains a plethora of conjugative transposons (CTns) and mobilisable transposons (MTns).

Usually these elements confer resistance to MLS$_B$ antibiotics (ermB) or tetracycline (tetM).

PLosOne. 2011. 8: e23014
CTns
capable of transferring themselves from a donor cell to a recipient using a conjugative-like mechanism

Tn916/Tn916-like elements:
Tn5397 (tetM)
Tn6194 (ermB)
CTn1/CTn1-like (catD, ABC transporter)
CTn6
CTn7

Tn1549-like elements:
CTn2
CTn4
CTn5/CTn5-like (ABC transporter)

MTns
lack genes for conjugation and use genes of CTns or conjugative plasmids present in the same cell

Tn5398/ Tn5398-like elements (ermB)
Tn6215 (ermB)
Tn6115 (ABC transporter)
Tn4453a/Tn4453b (catD)
<table>
<thead>
<tr>
<th>Tn5398 (MTn)</th>
<th>C. difficile</th>
<th>S. aureus</th>
<th>B. subtilis</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Tn5397 (CTn)</th>
<th>C. difficile</th>
<th>E. faecalis</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Other Tn916 –like (CTns)</th>
<th>C. difficile</th>
<th>B. subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLoS One. 2011. 6(8), e23014</td>
<td></td>
</tr>
</tbody>
</table>

The mobilizable non-conjugative element Tn5398 has been mainly detect in *C. difficile* RT012

AAC. 2005. 49:2550-2553
Microbial Drug Resistance. 2014. 20: 555-560

Tn916-like conjugative elements are widespread among *C. difficile* strains of different RTs

Research in Microbiology. 2015. 166:361-367
AAC. 2005. 49:2550-2553
Tn6194 is the most frequently found CTn ermB-containing element in EU epidemic RTs (including RT027, 001 and 017)

Nat Genet. 2012. 45:109-113
Microbial Drug Resistance. 2014. 20: 555-560

Tn6194-like element from CII7 (RT001) is able to in vitro transfer to C. difficile and E. faecalis JH2-2.
It integrates into the C. difficile recipients genome at different sites. A consensus 5’-C[TC]T[AG]GGAG[GA][GT]-3’ was identified.
Fitness cost of Tn6194-like element (strain CII7) and Tn5398 (strain 630) was investigated evaluating growth rates of transconjugants and recipient strain CD13 (RT039) or CD37 (RT009) and by competitive assays.

All transconjugants show growth rates significantly reduced.

In competitive assays transconjugants from recipient strain CD13 were disadvantaged, while those from CD37 were differently affected.

Independently of the burden that the element imposes on fitness, other factors (the capacity of in vivo transfer, the different insertion sites and the intrinsic characteristics of strains) are involved in the successful spreading and persistence of an element among C. difficile population.
Tn6215 and Tn5398 can integrate the genome of *C. difficile* through exchange of large genomic fragments.

*Mobile Genetic Elements. 2015. 5: 12-16*

Exchange of large genomic fragments has been also observed in *C. difficile*.

Pathogenicity locus (PaLoc) transfer

*NATURE COMMUNICATIONS. 2013. 4:2601*

Whole genome sequence comparison has demonstrated that exchange of large block of DNA between strains is an important driver of *C. difficile* genome evolution.

*PNAS. 2010. 107: 7527-32*

*NATURE COMMUNICATIONS. 2013. 4:2601*
Alteration in the target sites of antibiotics

**FQs resistance:**
alterations in the quinolone-resistance determining region (QRDR) of either GyrA or GyrB, the DNA gyrase subunits

**RIF resistance:**
alterations in the β-subunit of the RNA polymerase (RpoB)
Thr82Ile in GyrA is the most widespread among toxigenic clinical isolates resistant to FQs (93%)

Arg505Lys is the most widespread among RT027 (65%) Resistant to RIF


Substitutions in GyrA or GyrB

<table>
<thead>
<tr>
<th>RpoB amino acid substitution</th>
<th>Reduced rifaximin susceptibility</th>
<th>Ribotype (RT) (number of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>No</td>
<td>Different RTs (246)</td>
</tr>
<tr>
<td>R505K</td>
<td>Yes</td>
<td>RT027 (46)</td>
</tr>
<tr>
<td>T501T + L506L + G510G + G512G + F521F + E541E + K556K</td>
<td>No</td>
<td>RT027 (1) , RT126 (4)</td>
</tr>
<tr>
<td>HS02N + RS05K</td>
<td>Yes</td>
<td>RT045 (1) , RT061 (1)</td>
</tr>
<tr>
<td>A555A</td>
<td>No</td>
<td>RT027 (1) , RT03 (3)</td>
</tr>
<tr>
<td>HS02N</td>
<td>Yes</td>
<td>RT045 (1) , RT061 (1)</td>
</tr>
<tr>
<td>HS02L</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
</tr>
<tr>
<td>HS02Y</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
</tr>
<tr>
<td>S475S + F481F + D492D + T501T + A506A + G510G + T539Y + K556K + S575A</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
</tr>
<tr>
<td>L487F + HS02Y</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
</tr>
<tr>
<td>D492N</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
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<tr>
<td>HS02N + A555A</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
</tr>
<tr>
<td>RS05K + LS04M</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
</tr>
<tr>
<td>S550F</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
</tr>
<tr>
<td>S550Y</td>
<td>Yes</td>
<td>RT027 (1) , RT061 (1)</td>
</tr>
</tbody>
</table>

Substitutions in RpoB
FQ-resistant mutants were generated by introduction of point mutations into either the gyrA or gyrB QRDR domains of the strain *C. difficile* 630 using a two-step allele exchange.

Substitutions introduced:
- Thr82Ile (common)  Thr82Val (rare)  GyrA
- Asp426Asn (rare)  Asp426Val (rare)  GyrB

**Thr82Ile** had no detectable cost on the fitness

This substitution can be maintained in *C. difficile* population even in the absence of antibiotic selective pressure.
Resistance to antibiotics can be multi-factorial

Alterated expression of redox-active proteins, iron metabolism and DNA repair

Reduced susceptibility to MTZ

Sessile cells show increased resistance to MTZ

Alterated metabolic pathways involving pyruvate-ferrodoxin oxidoreductase
The separate acquisitions of fluoroquinolone resistance and a conjugative transposon in two distinct lineages of *C. difficile* 027/BI/NAP1 are the key genetic changes linked to its rapid emergence during the early 2000s.

"....the acquisition of resistance to commonly used antibiotics is a major feature of the continued evolution and persistence of *C. difficile* 027/BI/NAP1 in healthcare settings"
✓ *C. difficile* epidemiological changes are also associated to changes in antibiotic resistance

✓ National and international surveillances of antibiotic susceptibility patterns are needed

✓ Investigation on mechanisms responsible for antibiotic resistance

✓ New treatment strategies
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Nigel P. Minton
Sarah A. Kuehne
Stephen Cartman

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