

Chapter 2

Direct PCR-Ribotyping of *Clostridium difficile*

Sandra Janezic

Abstract

PCR-ribotyping, a method based on heterogeneity of ribosomal intergenic spacer region, is the preferred method for genotyping of *Clostridium difficile*. Standardly used procedure for PCR-ribotyping is culturing of *C. difficile* from fecal samples and subsequent typing. In this chapter, we describe a modified PCR-ribotyping method for direct detection of PCR-ribotypes directly in total stool DNA extract, without prior need to isolate *C. difficile*.

Key words Direct typing, PCR-ribotyping, Total stool DNA, *Clostridium difficile*

1 Introduction

Clostridium difficile infections (CDI) are a substantial burden to the health care system. Since the emergence of epidemic *C. difficile* strain of PCR-ribotype 027 that caused the first outbreaks in 2003 in Canada [1, 2] and has thereafter spread across North America and Europe [3, 4], outbreaks of CDI, which are mainly attributed, but not limited to the epidemic 027 strain, have been commonly present in hospital settings [5–10]. It is therefore important to identify epidemic strains as quickly as possible. Standard routine is to culture *C. difficile* from fecal samples and subsequent genotyping. For *C. difficile* different typing methods were developed and were reviewed by several authors [11–13]. The most commonly used method in Europe, and which has also been recently adopted in the USA, is PCR-ribotyping [14, 15]. The method is based on the amplification of intergenic spacer region (ISR) between 16S and 23S rRNA genes. The ISRs differ in length (200–600 bp) and in combination with variable number of alleles of the ribosomal operon present in different *C. difficile* strains PCR amplification of ISRs with only a single primer pair result in a banding pattern, specific for a given PCR-ribotype.

PCR-ribotyping approach was first described by Gürtler [16]. The method was then modified by O'Neill et al. [17] and was as

such adopted in Anaerobe Reference Unit, Cardiff, UK, for a routine typing of *C. difficile* [18]. Soon after, Bidet et al. [19] designed new primers which are located closer to ISR, yielding smaller amplicon sizes (Fig. 1). Both primer sets give comparable banding patterns and are routinely used in different laboratories.

We have recently described a new primer set that enables PCR-ribotyping of *C. difficile* directly in total stool DNA, therefore avoiding the need to culture isolates. New primers were designed to anneal partially within the 16S (forward primer) and 23S (reverse primer) rRNA genes and partially within conserved regions at 5' and 3' of ISR (Fig. 1), resulting in specificity for *C. difficile* (Fig. 2) [20, 21]. These primers give comparable results to primers described by Bidet et al. Only difference is in amplicon lengths (relative difference in fragment lengths of 24 bp), thus hindering direct comparison of profiles generated by different primer pairs.

Direct PCR-ribotyping is a rapid method and it gives information on *C. difficile* PCR-ribotype within a day, in contrast to conventional PCR-ribotyping of cultured isolates where results are available after 2–3 days, and can be convenient typing method in situations when only total stool DNA is available.

This chapter describes material and methods used for detection of *C. difficile* PCR-ribotypes directly in total stool DNA extracts.

2 Materials

2.1 Total Stool DNA Extraction

1. Commercial kit for total stool DNA extraction, such as QIAamp DNA Stool Mini kit (Qiagen) (*see Note 1*).

2.2 PCR Amplification

1. Primers for amplification of 16S-23S rDNA intergenic spacer region, ISR (5'–3'):
 - (a) 16S RT: GCTGGATCACCTCCTTTCTAAG (annealing on 16S rRNA gene and 5' end of ISR).
 - (b) 23S RT: TGACCAGTTAAAAAGGTTTGATAGATT (annealing on 3' end of ISR and 23S rRNA gene).
2. Taq DNA polymerase with MgCl₂ (Roche) and amplification buffer.

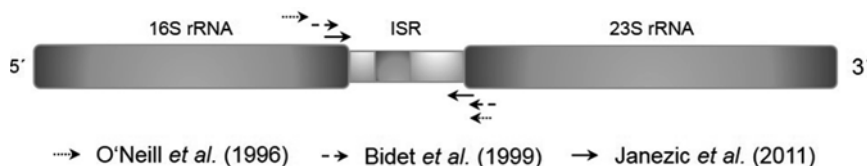


Fig. 1 Schematic representation of localizations of PCR-ribotyping primers within the *C. difficile* *rrn* operon

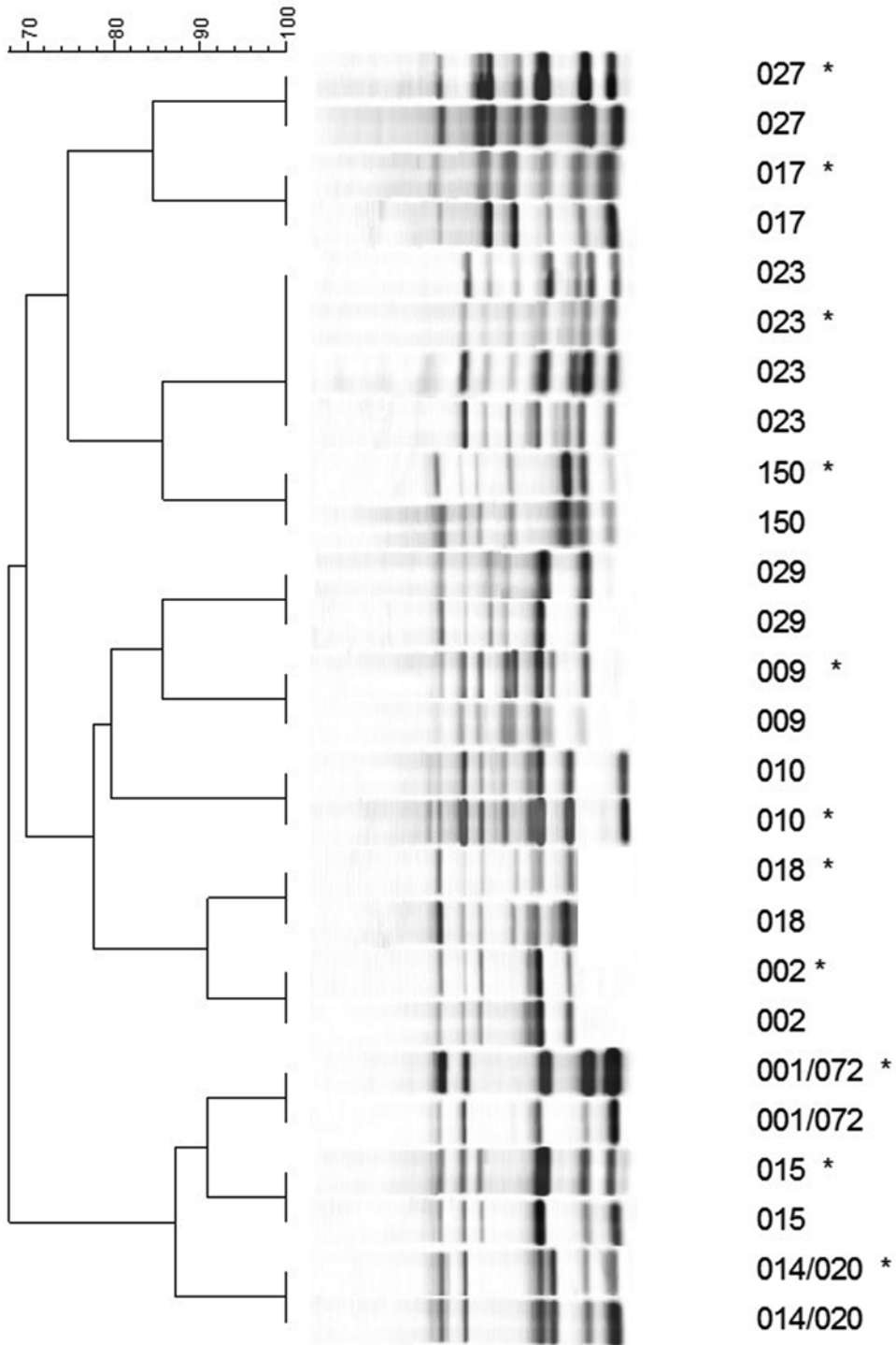


Fig. 2 Comparison of PCR-ribotyping patterns obtained from total stool DNA (marked with *) and reference strains using primers and protocol described in Janezic et al. [20] and in this chapter

3. dNTPs (Roche); working solution of 20 mM dNTPs mix (5 mM each dNTP).
4. Bovine serum albumin, BSA (10 mg/ml).

2.3 Agarose Gel Electrophoresis

1. Certified™ Low range Ultra Agarose (Bio-Rad) (*see Note 2*).
2. TAE buffer (50×): 2.0 M Tris-acetate, and 50 mM EDTA. Adjust pH to 7.0. For working solution (1×) dilute 20 ml of 50× TAE with 980 ml of distilled water. Cool the buffer to 4–8 °C before use (*see Note 3*).
3. Loading dye.
4. DNA staining solution: Ethidium bromide (EtBr) in distilled water with final concentration of 0.2 µg/ml.

2.4 Analysis of Banding Patterns

1. BioNumerics software (Applied Maths) (*see Note 4*).

3 Method

3.1 Isolation of Total Stool DNA

For isolation of total DNA from feces, use a commercial kit and follow the manufacturer's recommendation (*see Note 1*).

3.2 PCR Amplification

Prepare PCR master mix according to Table 1 and aliquot 45 µl of master mix in PCR tubes. Add 5 µl of DNA and run a PCR with the following amplification conditions:

- initial denaturation at 95 °C for 5 min,
- 35 cycles of:
 - 1 min at 95 °C for denaturation,
 - 1 min at 57 °C for annealing, and
 - 1 min at 72 °C for elongation,
- final elongation at 72 °C for 10 min.

After amplification, concentrate the PCR products by heating for 45 min at 75 °C. To let the water evaporate, open the caps on centrifuge tubes and leave the lid of thermocycler open.

3.3 Agarose Gel Electrophoresis

For separation of amplified ISRs prepare a 3% agarose gel (Certified™ Low Range ultra agarose; Bio-Rad; *see Note 2*) in 1× TAE buffer. In microwave oven melt the agarose with occasional gently mixing with magnetic stirrer; do not allow bubbles to form (*see Note 5*). If agarose starts to overboil, pause the microwave oven and let it to calm down and then continue with heating; repeat as many times as needed. Cool the agarose to 50–60 °C (in water bath with occasional gentle mixing on magnetic stirrer). Carefully (again avoiding bubbles) pour the agarose and let it to

Table 1 PCR mastermix composition

Reagent	Volume (μl)
PCR-grade water	36.0
10× PCR buffer (Roche) ^a	5.0
dNTPs (20 mM mix)	2.0
Primer 16S RT (50 μM)	1.0
Primer 23S RT (50 μM)	1.0
BSA (10 mg/ml)	0.5
DNA polymerase (5 U/μl)	0.25
DNA	5.0

^aFinal concentration of MgCl₂ in reaction mixture should be 1.5 mM

solidify for 30–45 min and run the electrophoresis at 2.5 V/cm for 4 h (*see* **Note 3**).

Load 20 μl of concentrated PCR product. It is important to include also a reference DNA standard in multiple positions in each gel (e.g., lanes 1, 7, 13, and 20 of a 20-lane gel) for normalization of a gel picture and accurate comparison of banding patterns.

To visualize DNA fragments, stain the gel in ethidium bromide (*see* **Note 6**) for 20–30 min and then de-stain in distilled water for 20–30 min. Capture the image with gel documentation system.

3.4 Analysis of Banding Patterns and Determination of PCR-Ribotypes

PCR-ribotypes can be determined by comparison of banding patterns with reference PCR-ribotypes using appropriate software (e.g., BioNumerics software; *see* **Note 4**) with in-house-built comparison library or, although not advisable and time consuming, manually by running reference strains of interest in the same gel. Isolates having identical banding patterns are considered to belong to the same PCR-ribotype. If two isolates differ in only a single band, they belong to different PCR-ribotypes.

4 Notes

1. Other commercial kits can be used for isolation of total stool DNA. A pre-step with mechanical disruption with the SeptiFast Lyse Kit on MagNA Lyser instrument (Roche) or equivalent can be included to improve the lysis of *C. difficile* cell wall.
2. Other agaroses, suitable for resolution of small PCR fragments, can be used.

3. Always use a fresh 1× TAE. A cold 1× TAE buffer should be poured to electrophoresis chamber just before loading the gel to avoid excessive heating of the buffer during electrophoresis, which can cause DNA degradation.
4. BioNumerics software (Applied Maths, Belgium) is most commonly used for analysis of banding profiles and setting up databases and reference libraries. However, other analysis software can be used as well.
5. A 3 % agarose suspension is very thick, so the stirring should be gentle (100–150 rpm). If using microwave oven for melting the agarose use only stirrers covered with plastic.
6. Ethidium bromide is a potential mutagen and must be handled with care; use only appropriate gloves and dispose the staining solution and gel appropriately.

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