Introducing the problem

*Clostridium difficile* is the primary etiologic agent of nosocomial diarrhoea (Barbut *et al.*, 1996) and is responsible for an important increase in hospital stays, with high healthcare and economic repercussions (Wilcox *et al.*, 1996).

Typing of the micro-organism can be useful in the detection and control of epidemic outbreaks and endemic situations, and in the characterization of recurrences (Alonso *et al.*, 2001; Kato *et al.*, 1996). Different approaches have been used for typing bacteria, although PFGE has traditionally been the gold standard (Finney, 1993). PFGE has proved to be discriminatory and reproducible for typing *Clostridium difficile*; however, a considerable proportion of strains are non-typable by this technique due to the degradation of the DNA during the procedure, making uninterpretable gel smears (Kato *et al.*, 1994; Bidet *et al.*, 2000). Different modifications of the typing procedure have been proposed (Corkill *et al.*, 2000), but they have led to variable results with only partial improvement (Klaassen *et al.*, 2002; Fawley & Wilcox, 2002).

The aim of this study was to develop a new PFGE protocol which can offer universal typability for all our *C. difficile* strains.

Methods

**Samples and tests.** Two hundred and twenty toxigenic *C. difficile* isolates were included in this study. We obtained isolates from the diarrhoeic stool samples of patients admitted to our hospital over a period of 6 months. Cultures, identification and toxin detection were carried out using standard procedures. The CHEF bacterial DNA plug kit (BIO-RAD) was used for PFGE typing the isolates. The standard manufacturer’s recommended technique and our modified protocol were performed and compared.

**Standard protocol.** Briefly, the recommended standard protocol was as follows. A bacterial culture was inoculated into LB broth with agitation and grown to an OD_{600} of 0.8–1.0. Then 5 x 10^7 bacteria were removed for each millilitre of agarose plug to be made and centrifuged to obtain the bacterial pellet. The supernatant was discarded and the pellet resuspended in 500 μl of cell suspension buffer. The bacterial suspension was warmed to 50 °C. The 2 % CleanCut agarose was melted and equilibrated to 50 °C. Five hundred millilitres of agarose were added to the bacterial suspension and mixed thoroughly. The mixture was transferred to plug moulds and the agarose was allowed to solidify at 4 °C for 10–15 min.

The solidified plugs were incubated in 1 mg ml$^{-1}$ lysozyme solution (100 μl 25 mg ml$^{-1}$ lysozyme stock plus 2.5 ml lysose buffer) for 2 h at 37 °C. The lysozyme was removed and the plugs were rinsed with sterile water. The plugs were incubated with >20 U ml$^{-1}$ proteinase K solution (100 μl of >600 U ml$^{-1}$ proteinase K stock in 2.5 ml of proteinase K reaction buffer) at 50 °C for 24–96 h. The plugs were washed four times in 1X wash buffer for 1 h each at room temperature with gentle agitation. The second or third wash contained 1 mM PMSF to inactivate residual proteinase K.

Prior to endonuclease restriction, each plug was washed for 1 h in 1 ml 0.1X wash buffer and then rinsed with fresh 0.1X wash buffer. The plug was incubated for 1 h with 1 ml of 1X restriction enzyme buffer at room temperature and then overnight in 300 μl of 1X restriction enzyme buffer containing 30–50 U of the restriction enzyme at the appropriate temperature. After overnight digestion, the buffer was removed and the plug was incubated for 30 min in 1X wash buffer and then equilibrated in the appropriate concentration of gel-running buffer (e.g. 0.5X TBE).

The manufacturer recommended loading one-quarter to one-third of a plug per well for electrophoresis. Although the manufacturer does not give any recommendation about the electrophoresis itself, most of the standard published series use 1 % agarose gels in 0.5X TBE buffer and the electrophoresis parameters vary widely depending on the restriction endonuclease used.

**Modified protocol.** Our protocol was based upon the standard commercial procedure as described, with the following modifications.

Fresh 24 h plate cultures were always used. When unfreezing strains, they were grown three times in CCFA or Brucella agar before starting.
BHI tubes were inoculated and incubated for 24 h to obtain a high-density inoculum (OD600 > 1.2). Incubations of more than 24 h were not performed. We removed 10⁹ bacteria for each millilitre of agarose plug to be made. A fresh preparation of the BHI culture was observed by microscopy at ×1000 magnification to check for the presence of spores. Cultures with a high proportion of spores (over 40%) were ruled out. Lysozyme and proteinase K (Sigma) were prepared 'in-house' and used at high concentrations (2 mg ml⁻¹ and 75 U ml⁻¹, respectively). Proteinase K incubation was no longer than 18 h. Washing steps were reduced to 30 min. Endonuclease digestions were performed with high concentrations of enzyme (Smal, 60 U in 300 μl volumes). Thiourea (Sigma), always freshly prepared and kept in darkness until use, was included in the gel and running buffer in high concentrations (200 μM).

Electrophoresis. Electrophoresis was run at 195 V for 20 h with pulse times starting at 4 s and ending at 30 s. Gels were stained with ethidium bromide, visualized under UV transillumination and recorded.

Results and Discussion

Using the standard procedure, almost 60% of the assayed isolates (130) remained untypable by PFGE (Fig. 1). The remaining strains offered good PFGE patterns that were reproduced on every occasion (e.g. the reference strain, C. difficile ATCC 9689, was included in every run and was always correctly typed).

Although most of our non-typable isolates (98, 75%) belonged to our most prevalent ribotype (R1), a considerable proportion (32, 25%) were from several others (data not shown). Some other authors have reported the same phenomenon with PFGE and C. difficile at different frequencies. In most cases, non-typable strains belonged to serogroup G and ribotype 1 (O’Neill et al., 1993; Kato et al., 1996) (some strains from this serogroup are typable; Bidet et al., 2000), although others have also reported the problem in different strains (Kato et al., 2001; Spigaglia et al., 2001). Unfortunately, we did not serotype our strains and we used a different ribotyping scheme (Bidet et al., 2000), as a result of which we do not know whether our non-typable strains belong to those reported major groups.

With the described modifications to the protocol we increased the typability of the technique, enabling all our C. difficile isolates to be characterized by PFGE (Fig. 1b).

Several factors have been proposed to explain the lack of typability in C. difficile by PFGE. Firstly, the micro-organism has very potent endonucleases that may degrade the extracted DNA inside the agarose plug during the extended process (Spigaglia et al., 2001). Some strains may produce fewer nucleases, which could be removed or degraded during nucleic acid extraction. In our proposed protocol, we shortened incubation and washing times in order to avoid or reduce such degradation. The high concentrations of proteinase K used could also help to digest nucleases more efficiently.

Secondly, it has been reported that a nucleolytic peracid derivative of Tris may form at the anode during electrophoresis and this chemical nucleolysis could be inhibited by the addition of thiourea to the running buffer (Ray et al., 1995). This effect has also been described for other micro-organisms (e.g. Pseudomonas aeruginosa; Römling & Tümmler, 2000). The concentration of thiourea is controversial, and while some authors defend that 50 μM is enough (Corkill et al., 2000), others report concentrations of up to 200 μM in both the gel and the buffer (Fawley & Wilcox, 2002). We found that 50 μM in the buffer had a reduced effect whereas the best results were achieved with 200 μM in gel and buffer.

Thirdly, we think it is extremely important to avoid spore formation. Cultures with a high rate of sporulated forms probably exhibit poorer lysis and produce less DNA. For this reason we recommend working with fresh cultures and screening for the presence of spores by microscopy before starting the DNA-extraction process. We also recommend using high lysozyme and proteinase K concentrations to facilitate spore lysis. Hypothetically, the strains belonging to our major ribotype could be more likely to produce spores, which could explain both the difficulty in PFGE typing and a favourable spread in the hospital. According to this, many outbreaks have been reported to be caused by PFGE non-typable strains (Kato et al., 2001; Samore et al., 1997).

In conclusion, the above-mentioned modifications enabled us to achieve complete typability (increasing from 40% to 100%) within the assayed isolates while maintaining both a high degree of discrimination and the reproducibility of the technique.

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References


