Overview report on the application of double digest selective label (DDSL) bacteria genotyping technique for identification of strains and certification of commercially used bacteria

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Abstract

The objective of this report is to demonstrate the potential of the proposed simple typing technique, double digest selective label (DDSL), which was initially developed to identify clinical isolates of *Pseudomonas aeruginosa*, for other bacterial species including *Salmonella enterica*, *Clostridium difficile*, *Staphylococcus aureus*, and *Bacillus subtilis*. The technique is based on digestion of bacterial genomic DNA with two restriction enzymes and simultaneous labeling fragments with biotinylated deoxycytidine triphosphate in fill-in reaction by *Taq* polymerase. The number and distribution of generated DNA fragments can be optimized by selecting restriction enzymes. DDSL is fast, reproducible, cost effective and sufficiently discriminatory typing method applicable for identification of bacterial strains at laboratories having no access to expensive sequencing equipment and with limited funding and lack of skilled personnel. Data concerning the potential of the technique for short-term epidemiological surveillance and bacterial strain certification are presented and discussed. Multiple locus variable number tandem repeat analysis performed on our set of *Clostridium difficile* isolates did not demonstrate sufficient discriminatory power both with TR6 and TR10 loci on a set of 24 isolates. In contrast, the DDSL analysis resolved all isolates into individual strains.

Keywords Bacteria · Genotyping · Restriction enzymes

Introduction

Epizoonotic outbreaks pose a serious threat to human health and animal welfare. Contagious infectious diseases are currently the focus of increasing interest due to globalization of food distribution, the potential for bioterrorist attacks and the spread of antibiotic resistant strains. Bacterial typing is a key technology in human and veterinary medicine, community health, consumer protection and in agricultural research. The importance of the development of epidemiological tracking tools is underlined by numerous outbreaks of diseases due to bacterial pathogens, which are a significant economic and health problem in many countries. Particularly important is

tracing pathogen dissemination in “real time”, i.e., to use a fast typing technique to distinguish between clonally related (epidemic) strains and unrelated (sporadic) strains in a short-term epidemiological setting to follow the chain of pathogen transmission and to localize potential reservoirs. Currently, whole genome sequencing (WGS) is gaining popularity as a typing tool for tracking pathogen dissemination in both community and global settings as well as for simultaneous detection of virulence and antibiotic resistance genes. The WGS data is primary managed by SNP and/or gene-by-gene (cgMLST) comparisons in reference laboratories (Schürch et al. 2018). Nevertheless, pulsed-field gel electrophoresis (PFGE) is still considered as a valuable typing method for short-term studies with many bacterial species including *Salmonella* spp., *Clostridium difficile* and *Staphylococcus aureus* (Broukhanski et al. 2011; Walters et al. 2013) and applied for the internationally recognized PulseNet database. In contrast to PFGE, WGS data is not suitable for establishment of general relatedness guidelines. WGS differences between strains are considered on case-by-case basis and attempts to establish species-specific cutoffs are unlikely

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to be generally applicable. Currently, there are discussions which part of bacterial genome (core, accessory, regulatory or pan-genome) should be analyzed to get clear answers to specific epidemiological questions (Schürch et al. 2018). Wide application of WGS is restricted by high cost of the equipment and requirement for the skilled personnel to interpret generated sequence data. That is why, gel-based techniques is still in high demand, particularly in low-income countries. Two disadvantages of PFGE typing are the long analysis time (Hardy et al. 2012) and use of expensive specialized equipment (pulsed-field system). Widely used multilocus sequence typing (MLST) and multiple locus VNTR analysis (MLVA) which proved to be useful for a number of bacterial species, for some applications show poor discrimination in short-term settings (Werner 2013; Hardy et al. 2012) and require application of PFGE and MLVA, or PFGE and MLST in tandem to get sufficient discriminatory power (Rumore et al. 2016; Turki et al. 2014). Summarizing, none of the currently existing techniques for pathogenic bacteria typing in a short-term setting fully meets all performance requirements and there is still a demand for a fast, cost effective and discriminative method (van Belkum 2007). Previously obtained data showing the feasibility of double digest selective label (DDSL) typing for *Pseudomonas aeruginosa* clinical isolates (Terletskiy et al. 2008) was used as the basis for optimization of the method so that it can be used for sets of bacterial isolates of other bacterial species. It significantly improves speed and accuracy of epidemiological surveys aimed at curbing infectious diseases and identifying the sources of pathogens. The objective of this article is to demonstrate the potential of this fast typing technique for several bacterial species. In the report, the method has been optimized for epidemiological analysis of clinical isolates of *Salmonella enterica*, *Clostridium difficile*, and *Staphylococcus aureus*. Besides, the approach has now been applied at our laboratory for strain confirmation (certification) of *Bacillus subtilis* strains which form the basis for commercially used biopreparations against phytopathogens of bacteria and fungi origin.

Materials and methods

Underlying concept of DDSL

The underlying concept of the DDSL is that the large macrorestriction fragments currently produced by the rare cutting restriction enzyme can be reduced in size by simultaneous digestion with a second frequently cutting restriction enzyme to give a large number of smaller fragments that can be rapidly separated on a conventional agarose gel. In the DDSL approach, the large macrorestriction fragments produced by *Xba*I enzyme with *Salmonella enterica* DNA,

*Spe*I (*Pseudomonas aeruginosa*), *Cfr*9I (*Staphylococcus aureus*), *Mlu*I (*Clostridium difficile*), and *Sgs*I (*Bacillus subtilis*) normally analyzed by PFGE are trimmed by a second enzyme to give smaller fragments. The rare cutters (“detection” enzymes *Xba*I, *Spe*I, *Cfr*9I, *Mlu*I, and *Sgs*I) producing 3'-recessed ends will give a limited number of extremely long fragments which can be tagged with biotinylated deoxycytidine triphosphate (Bio-dCTP) in a fill-in reaction with the standard *Taq* polymerase. Computer analysis of the published genomic sequence (<http://insilico.ehu.es/diges/t/>) predicts that, in case of *Salmonella Typhimurium* strain LT2 and *Xba*I enzyme, only 27 fragments will be produced. Comparable number of fragments are produced with *Spe*I, *Cfr*9I (isoschizomer of *Sma*I), *Mlu*I, and *Sgs*I enzymes in corresponding genomes. Most of these fragments are too large for separation by standard agarose gel electrophoresis. For this reason, we propose the addition a second frequent cutting “trimming” enzyme in a single-tube double-digestion reaction. Frequent cutters digesting genomic DNA at 800–2000 sites (*Pst*I for *S. enterica*, *Eco*147I for *P. aeruginosa*, *Hae*II for *S. aureus*, *Mph*110I for *C. difficile*, and *Eco*32I for *B. subtilis* isolates) will ensure obtaining relatively short DNA fragments for further separation in a standard agarose gel. Intrinsic feature of all frequent cutters are that they produce either blunt or 3'-protruding fragment ends, which cannot incorporate Bio-dCTP tag. Other criteria to select frequently cutting enzymes are restriction buffer compatibility with rare cutters, high specificity, cost, commercial availability, and they should not be prone to star activity. The DDSL genotyping protocol produces similar data set as seen with the currently accepted standard PFGE method based on macrorestriction digestion, because the same “rare cutting” enzymes are used in both methods.

In this report, the method has been adapted for epidemiological analysis of clinical isolates of *Salmonella enterica* (Collection of bacterial cultures kept at the University of the Basque Country, Vitoria-Gasteiz, Spain), *Clostridium difficile* (clinical isolates collected in 2008, CHUV, Switzerland), and *Staphylococcus aureus* (the set of isolates collected from pet animals kept at German veterinary clinic (Strommenger et al. 2006). Besides, the approach has been applied for strain confirmation (certification) of *Bacillus subtilis* strains (Bacterial Bioresource Collection, Institute of Plant Protection, Russia), which form the basis for commercially used biopreparations against phytopathogens.

Technical details of DDSL

The main steps in the DDSL approach are as follows:

1. **Digestion.** Simultaneous digestion of the bacterial genomic DNA (0.5–1 µg) with a rare cutting “detection” and a frequently cutting “trimming” enzymes (5 U each)

in a single tube and a single buffer results in numerous restriction fragments. Simultaneous labeling with Bio-dCTP (0.2 µl for 20 samples) using fill-in reaction generates limited number of biotinylated fragments. It is crucial to use low amount of *Taq* polymerase (<0.1 units per reaction) to avoid non-specific incorporation of Bio-dCTP in the CCGG, CGCG and CTAG 3'-recessed ends produced by rare cutting enzymes. Digestion/labeling reaction requires 1 h at 37 °C to complete. Alternatively, fast digest protocol which requires only 5 min can be applied. The final volume of the reaction mix is 20 µl.

2. **Electrophoresis.** Fragments produced after double digestion/labeling are subjected to 4 h electrophoresis at 150 V in 0.8% agarose with 20 cm long gels using 1xTAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and standard electrophoretic chamber and then immediately transferred to a Hybond N nylon membrane (GE Healthcare) at 40 mbar for 30 min using water as a transfer media and vacuum blotting device VacuGene XL Vacuum Blotting System™ (GE Healthcare, USA). The technique does not involve hybridisation step; therefore, there is no need for gel denaturation/neutralisation steps as in traditional Southern blotting/hybridisation.
3. **Detection of labelled fragments.** Visualization of labelled fragments by colorimetric detection based on alkaline phosphatase conjugated streptavidin (Str-AP) and chromogenic substrates (NBT and BCIP) requires 60 min and is performed according to established manufacture's protocol (Roche™).
4. **Analysis of band distribution.** This step can be performed by either manual visualization of bands or by application of specialized software such as Bionumerics to get more objective interlane comparisons and strain clustering data.

Multiple locus variable analysis (MLVA)

In addition to genotyping using DDSL method, a set of *C. difficile* isolates was typed by MLVA. This analysis was performed on the same isolates using the approach described in 2009 (Zaiss et al. 2009). The following primers were used to amplify polymorphic tandem repeats:

TR6-F 5'-TTTCAACTTGTCAGTTTTTAAGTC-3'
 TR6-R 5'-ATGACATAGCGTTTGTGGAAT-3'
 TR10-F 5'-TGCATCAAATTGGTCAAGACTC-3'
 TR10-R 5'-TGAAATCATTGACTATAAAGCAAAA-3'.

DNA amplification was performed on 1 µl of genomic *C. difficile* DNA in a final volume of 25 µl containing 0.1 µM of TR6 and TR10 primers, 200 µM of each deoxynucleoside triphosphate, 1×PCR buffer and 1 unit of Hot Start *Taq* DNA Polymerase (Invitrogen™). After an initial

denaturation of 96 °C for 3 min, the protocol consisted of 35 cycles at 96 °C for 45 s, 52 °C for 45 s, and 72 °C for 45 s following a final extension at 72 °C for 7 min. The PCR products were separated in 2% agarose gel, stained with fluorescent dye GelRed™ (Bio-Rad™), and photographed in a gel documentation device. GeneRuler™ 100 bp Plus DNA Ladder (Thermo Fisher Scientific™) served as a molecular mass marker.

Results and discussion

The DDSL technique generates 17–50 clearly distinguishable DNA fragments, the number and distribution of which depend on bacterial isolate, species and pairs of restriction enzymes used (Figs. 1, 2). PFGE permits the separation of large restriction fragments over a period of 24 h or more, while the new technique, DDSL, trims these large fragments to a size which can be separated on a conventional agarose gel within 4 h (Terletskiy et al. 2008). The fragment labeling by the *Taq* polymerase fill-in reaction was fast and could be performed in the same reaction buffer and microtube as the digestion itself. An additional advantage of DDSL is that individual bands are significantly sharper (Fig. 3) and there is a potential to resolve more bands than is possible with PFGE. In the latter typing technique the final banding pattern is not sometimes reproducible due to intrinsic PFGE sensitivity to such factors as efficiency of intra-gel intact DNA extraction and release of endogenous endonucleases. Typically, prolonged PFGE process results in generation of fuzzy bands which may limit the discriminatory power. In general, analysis of data shows that DDSL typing demonstrated at least the same discriminatory power and similar strain clustering as those of PFGE performed on the same set of isolates (Fig. 1 in Terletskiy et al. 2008).

The gain in efficiency is critical for rapid identification of the source of an infection and any further advance in efficiency would be valuable. An additional advantage of DDSL is that there is a potential for analysis of more bands than can be seen with PFGE, which means that the discrimination power is potentially increased. DDSL has the potential of giving more detailed information about genomic polymorphism and mutation rates in the bacterial species as it can detect small insertions and deletions. Sensitivity of some restriction enzymes to methylation of recognition sequences provides additional advantage of DDSL as this intrinsic feature of the technique makes it possible to discriminate bacterial strains with the same sequence of nucleotides but differing in their methylation status. Thus, the method provides microbiological laboratories having no access to capillary electrophoresis and high throughput separation technologies, with a reliable tool for tracking the spread of pathogenic microbes in short-term epidemiological studies.

Fig. 1 Representative DDSL banding patterns obtained on sets of clinical bacterial isolates of *C. difficile*, *P. aeruginosa*, *S. enterica*, and six commercially used *B. subtilis* antagonistic strains. Images

from separate DDSL experiments on different bacterial species are combined in one figure

Modern approaches based on sequencing (e.g., NGS) provide plethora of information on genetic variations, presence of antibiotic genes, virulence factors etc. These data can be used for epidemiological studies and normally released by national, local official and reference laboratories. DDSL data can be generated by laboratories having no access to specialized and expensive sequencing equipment primarily for short-term epidemiological observations when it is necessary to differentiate between epidemic and sporadic bacterial strains or to identify routes of pathogen transmission. There is a potential improvement of the DDSL technique when instead of agarose gel separation and biotin detection chemistry, one can use Agilent Bioanalyzer 2100 equipment with DNA 12000 LabChip kit and fluorescently labelled dCTP to get DDSL genetic profile of bacterial strains. In this case turn-around genotyping time can be reduced to only 20 min (provided that bacterial DNA is available and fast digest restriction enzymes are used).

Although the idea of using the trimming enzyme seems rather straight forward, experience has shown that the technical problems of combining two restriction enzymes and obtaining a clear banding pattern requires considerable experimentation with different restriction enzymes, buffers,

concentrations of *Taq* polymerase enzyme and biotinylated dCTP and the choice of enzymes appears to be specific for each species of bacteria.

In our hands, MLVA genotyping with TR6 primers did not show variation between *C. difficile* isolates except 27530 and 27531, which were also significantly different in the DDSL picture (Fig. 2). Similar limited variation was also detected using TR10 primers. This observation is contradictory to conclusions made by other authors (Zaiss et al. 2009). In that paper authors claim that investigating a panel of 154 *C. difficile* isolates by MLVA, extensive sequence variation between isolates by both TR6 and TR10 loci was obtained. To explain these results we can assume that MLVA typing may be sensitive to origin of *C. difficile* sets of isolates and discriminatory power may vary from one set to another. In contrast, DDSL typing consistently provides high discrimination on a variety of sets of isolates regardless of their origin.

As DDSL technique is based on a simple restriction digestion of genomic DNA and simultaneous fragment labeling, the generated genetic profiles are highly reproducible. *Taq* polymerase is still able to efficiently incorporate a single Bio-dCTP molecule into 3'-recessed fragment

Fig. 2 DDSL genotyping of 24 hospital *C. difficile* isolates (upper part); MLVA genotyping of *C. difficile* isolates by PCR using TR6 primers (lower part, shown only a section of the gel with two deviant strains—27530 and 27531, the rest were identical). Upper and lower parts of the figure are grouped images from different DDSL and MLVA experiments. R—reference strain No 27532

ends at 37 °C (although at 10% activity), the observation that makes it possible to perform digestion and labeling in a single reaction microtube at this temperature. Advantages of DDSL typing are speed of analysis, a potential for analysis of more bands than can be seen with PFGE (bands are sharper) and no requirements for expensive specialized equipment. Application of enzymes sensitive to methylation of recognition sequences can make the technique more discriminative, because in this case, it will recognize

strains with the same nucleotide sequences but different in distribution of methyl groups in bacterial genomes.

Conclusion

The DDSL typing technique is simple to perform, low cost, fast, discriminatory, and can be used as an alternative or supplementary to currently accepted methods for strain

Fig. 3 Representative DDSL pattern generated on a set of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (from Strommenger et al. 2006) with the *Cfi9I*+*HaeII* restriction enzyme combination

identification when it is necessary to discriminate between sporadic and epidemic infectious disease cases. This technique may also provide a suitable tool for a fast and cost effective certification of bacterial strains of *B. subtilis* (bio-control agent) which serve as a basis for formulation of commercial biopreparations used in plant protection schemes. The method is affordable at laboratories in low-income countries having no or limited access to sophisticated sequencing equipment.

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Author contribution V. Terletskiy put forward the concept of using two restriction enzymes simultaneously with selective labeling for genotyping and participated in all stages of the technique development and application. The author prepared all parts of the manuscript.

Compliance with ethical standards

Conflict of interest The author declares no conflicts of interest in the publication.

Ethical approval This article does not contain any studies with human participants or animals performed by the author.

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