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APPLICATION OF DNA TYPING FOR IDENTIFICATION OF BACTERIAL STRAINS INFECTING ANIMALS

Discovery notes

Abstract

Typing of an important pathogen, *Staphylococcus aureus*, permits epidemiological investigations within a community and within an individual hospital. Pulsed field gel electrophoresis (PFGE) is the current “gold standard” method for typing this bacterial species in short-term epidemiological studies. However, PFGE requires specialized equipment and is rather slow. This article demonstrates the potential of a new typing method (double digest selective label – DDSL) as a fast, highly discriminatory alternative to PFGE for the typing of *Staphylococcus aureus* isolates. In this new typing method, large DNA fragments are produced with a restriction enzyme commonly used for PFGE but are trimmed by a second enzyme to a size which can be separated on a conventional agarose gel within a short period of time. Selective labelling of a subset of the numerous restriction fragments gives a distinct banding pattern for each isolate. The advantages of DDSL typing are its speed of analysis (one day) and the potential for greater discrimination between isolates than can be achieved with PFGE typing. An online service allows simulating the technique and a suggestion tool is developed for easy selection of endonucleases for this genotyping method.

Keywords: *Staphylococcus aureus*, isolates, genotyping technique, pathogen.

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ИСПОЛЬЗОВАНИЕ ДНК-ТИПИРОВАНИЯ ДЛЯ ИДЕНТИФИКАЦИИ БАКТЕРИАЛЬНЫХ ШТАММОВ, ИНФИЦИРУЮЩИХ ЖИВОТНЫХ

Исследовательские заметки

Аннотация

Типирование важного патогена, золотистого стафилококка, позволяет проводить эпидемиологические исследования как в условиях клиник, так и вне медицинских учреждений. Пульс-гель электрофорез (ПГЭ) является «золотым стандартом» типирования этого вида бактерий в краткосрочных эпидемиологических исследованиях. Тем не менее, ПГЭ требует наличия специального оборудования и выполняется в течение длительного времени. Эта статья демонстрирует потенциал нового метода типирования (двойное расщепление и избирательное мечение – ДРИМ) в качестве быстрой и чувствительной альтернативы ПГЭ для типирования изолятов *Staphylococcus aureus*. В новом методе большие фрагменты ДНК получают с помощью фермента рестрикции, обычно используемого для ПГЭ, но, одновременно, расщепляют вторым ферментом до размера, который можно разделить в обычном агарозном геле в течение короткого периода времени. Избирательное мечение части из множества рестрикционных фрагментов дает четкую картину для каждого изолята. Преимуществами ДРИМ-типирования являются его скорость анализа (один день)

и возможность большей дискриминации между изолятами, чем это может быть достигнуто с помощью ПГЭ-типирования. Онлайн-сервис, позволяет смоделировать расщепление для быстрого выбора эндонуклеаз, используемых в генотипировании.

Ключевые слова: золотистый стафилококк, изоляты, метод генотипирования, патоген.

1. Introduction

Outbreaks of infectious diseases pose a serious threat to human health and animal welfare. Strain discrimination obtained by molecular typing techniques has become an essential tool for monitoring and preventing the transmission of pathogens such as *Staphylococcus aureus* [1]. The importance of epidemiological tracking tools is underscored by numerous studies of *Staphylococcus aureus* transmission both in hospital and community settings. In practice, it is particularly important to be able to trace pathogen dissemination in "real time" and this depends on the use of a fast typing technique which is available on site and which has sufficient precision to discriminate between clonally related (epidemic) and unrelated (sporadic) strains and to follow the chain of transmission and localize pathogen reservoirs [2]. Pulsed field gel electrophoresis (PFGE) is currently the “gold standard” typing method for short-term micro-epidemiological investigations. PFGE is suitable for many different bacterial species but each application requires careful optimization. For example, PFGE analysis of S*taphylococcus aureus* [3; 4] is currently based on the rare-cutting restriction enzyme, *Sma*I, which is accepted as being the most informative of several alternatives. A higher level of discrimination than that afforded by PFGE analysis would be beneficial in some studies and in other cases, it would be a great advantage to be able to perform an analysis in less than the several days which is typical for PFGE typing. A further disadvantage of PFGE is the specialized equipment it requires, which is not available in many hospitals. In some bacterial species, individual strains can not be typed by PFGE because of the expression of endogenous endonucleases [6]. Multilocus sequence typing (MLST) is a practical alternative but only for long-term studies because its level of discrimination is not sufficient to use in short-term settings where sequence differences are reduced [7; 8]. MLST is relatively expensive and screens only a preselected subset of conserved genomic sequences whereas whole genome typing methods (like PFGE) screen sites throughout the bacterial genome and are thus more reliable for studying pathogen diversity. In summary, none of the currently existing techniques for typing pathogenic bacteria fully meets all requirements for dependable short-term epidemiological analysis and there is an urgent demand for a rapid, cost effective alternative.

The objective of this article is to demonstrate the potential of new fast typing technique, double digest selective label (DDSL), which was initially developed to identify clinical isolates of *Pseudomonas aeruginosa*, another important nosocomial bacterial species. In this report, the method has been adapted for epidemiological analysis of *Staphylococcus aureus* and tested on two sets of isolates.

The underlying concept of the DDSL approach is that the large macrorestriction fragments currently produced by the restriction enzyme *Cfr*9I (an isoschizomer of *Sma*I which is used for PFGE typing of *Staphylococcus aureus*) can be reduced in size by simultaneous digestion with a second restriction enzyme, *Hae*II, to give a large number of smaller fragments that can be rapidly separated on a conventional agarose gel (Figure 1). The 3’-recessed ends of cuts produced by *Cfr*9I are suitable for end-labelling [9] with DNA polymerase whereas the 3’-protruding ends of cuts produced by the trimming enzyme (*Hae*II) are not labelled. The product of this single tube/single buffer digestion/labelling reaction is a characteristic set of labeled fragments suitable for rapid electrophoretic separation and detection. The result is a “barcode” banding pattern which identifies each bacterial isolate and is the key to rapid epidemiological analysis. An additional advantage of DDSL is that individual bands are significantly sharper and there is a potential to resolve more bands than is possible with PFGE.

2. Methods

2.1. Strains and DNA isolation

Two sets of *Staphylococcus aureus* isolates were used in this study. The first set included 15 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates collected at the diagnostic laboratory of the Institute for Microbiology and Infectious Diseases of the School of Veterinary Medicine (Hannover, Germany) from pet animals admitted to the veterinary hospital for stationary treatment [10]. The other set included 62 isolates obtained from milk samples of dairy cows kept in various herds of southern Brazil and suffering from subclinical mastitis.

Staphylococcal chromosomal DNA was extracted from 5 ml overnight cultures in BHI (brain heart infusion) growth medium. Bacterial lysis was accomplished with lysostaphin and sodium dodecylsulfate (SDS). DNA isolation was based on a single round of conventional phenol-chloroform extraction followed by isopropanol precipitation.

2.2. Digestion / labelling

Simultaneous digestion and labelling of the bacterial genomic DNA (0.5 -1 µg) was performed with 5 units of rare cutting *Cfr*9I (an isoschizomer of the *Sma*I used in PFGE) (GE Healthcare), 5 units of a frequent cutting enzyme, *Hae*II (New England Biolabs GmbH, Frankfurt, Germany), 0.2 units of Taq polymerase and 0.2 µM of biotinylated dCTP (Bio-dCTP, GE Healthcare) in a single tube and buffer (Tango^{TM}, GE Healthcare) (Figure 1). The reaction volume was adjusted to 20 µl with distilled water. The reaction time was 60 min, and the incubation temperature was 37°C.

With multiple bacterial isolates it was technically convenient to prepare a pre-mix containing the required amounts of two restriction enzymes (*Cfr*9I and *Hae*II), Taq DNA polymerase and Bio-dCTP. In this case, the protocol is significantly simplified, requiring only the addition of 1 µl of this mixture to each sample of chromosomal bacterial DNA in Tango^{TM} buffer. The pre-mix is stable for at least 1 month at – 20°C (storage for a longer period has not been tested).

2.3. Electrophoresis

Prior to electrophoresis, the reaction product was desalted using reusable microcolumns filled with G50 Sephadex® matrix (GE Healthcare). Electrophoretic conditions were as follows: 1xTAE, 20 cm 0.8% agarose gel, 5V/cm voltage. Conventional "submarine" agarose gel electrophoresis required approximately 3 hours for satisfactory separation of digested and labelled DNA fragments.

2.4. DNA TRANSFER

Once electrophoresis was completed, the separated fragments were directly transferred to a neutral nylon membrane (GE Healthcare). The DDSL technique does not involve hybridization so denaturation and neutralization of the DNA in the agarose gel is not required. Transfer to a nylon filter was performed in deionized water using a vacuum blotter (Amersham) for 30 minutes.

2.5. Detection

After transfer, the biotinylated DNA fragments were cross-linked to the membrane by UV-irradiation then visualized by application of Streptavidin-AP conjugate (170-3554, Bio-Rad, Munich, Germany) followed by a colorimetric reaction based on the alkaline phosphatase. Briefly, the filter was incubated for 10 minutes in blocking solution (0.1 M maleic acid, 0.15 M sodium chloride, 1x Roche blocking reagent™ - Roche Diagnostics GmbH, Mannheim, Germany -, pH 7.5) and then treated with Streptavidin-AP diluted 1:3000 in the blocking solution. Unbound conjugate was removed by two 10 minutes washes with maleic acid buffer (0.1 M maleic acid, 0.15 M sodium chloride, pH 7.5). After a short equilibration in alkaline buffer (0.1 M Tris, 0.1 M sodium chloride, pH 9.5), the filter was placed in a plastic dish containing developing solution consisting of the two chromogenic substrates NBT and BCIP in alkaline buffer. Colour development was normally complete within 15 minutes. Images were captured with a CCD camera or a flatbed scanner and saved as TIFF files for further evaluation.

2.6. Simulation of DDSL and suggestion tool

A program for simulation of DDSL was developed, and it is available at http://insilico.ehu.es/DDSL. The program is located in a server with Linux/Apache/MyQSL, and the scripting language used to develop the program was PHP. The program allows simulation of the DDSL against all up-to-dated sequenced prokaryotic genomes. Genomes were obtained from NCBI. A suggestion tool was also created, which allows selecting endonuclease combinations capable to generate a band pattern by DDSL. All data available through this tool has been pre-computed. The procedure to search this database is simple: user must select the genera of the prokaryote, the visualization method for bands (capillary electrophoresis or agarose gel) and the labelled nucleotide to be used in the experiment. In the first step, the service will show a table with endonucleases yielding fragment ends that may be labelled with the selected nucleotide. Those enzymes can be combined with additional endonucleases to yield bands by DDSL. After selection of one of the enzymes in this first table, a second table is shown containing all endonucleases which may be combined with the previous one. In the table the number of theoretical bands for each sequenced genome is shown. The number of searchable genomes in the database was 675 when this paper was written.

3. Results

Several trimming enzymes were evaluated on the basis of their cutting frequencies, and DDSL was assayed online prior to wet experiments with the simulation program created during this work (http://insilico.ehu.es/DDSL) . Besides cutting frequencies, other criteria were taken into account including buffer compatibility for a double digestion, the ability to produce highly specific cuts even at high excess over template DNA and optimal fragment / band distribution in an agarose gel. Optimal fragment size and relatively even band distribution for *Staphylococcus aureus* chromosomal DNA was eventually achieved using the combination of *Cfr*9I (rare cutter) and *Hae*II (frequent cutter, trimming enzyme). According to the enzyme selection program (http://insilico.ehu.es/digest) developed by Bikandi *et al.* [11], *Hae*II and *Cfr*9I produces 821 and 25 cuts in chromosomal DNA of the Mu50 strain of *S. aureus*, respectively. Taking into account the genome size of this bacterial species (approximately 2.9 mbp), the average size of detected fragments after double digestion with *Cfr*9I/*Hae*II restriction enzymes is expected to be approximately 1,800 bp. Less frequently cutting trimming enzymes resulted in band crowding in the upper part of the membrane (higher molecular mass fragments) whereas more frequently cutting enzymes produced too many small fragments which ran off the gel under the conditions used.

DDSL fingerprinting with *Cfr*9I/*Hae*II enzymes performed on 62 *S. aureus* isolates revealed 38 different patterns comprised of 28 - 33 fragments and ranging in size from approximately 15 000 bp down to 500 bp (Figure 1). Bacterial isolates were selected from previously characterized samples (PFGE clusters 1, 3, 4, 5, 6, 7, 8, 12 and 15) [12]. Isolates within these PFGE clusters were further resolved by DDSL typing. Cluster 1 (lanes 1 and 2); cluster 3 (lanes 3 and 4); cluster 4 (lanes 5-8); cluster 5 (lanes 9 and 10); cluster 6 (lanes 11 and 12); cluster 7 (lanes 13 and 14); cluster 8 (lanes 15-19); cluster 15 (lanes 20 and 21); cluster 12 (lanes 22 and 23). In contrast, samples in lanes 6, 12 and 18 show the same DDSL patterns but these could be resolved by PFGE typing into individual strains [12].

In comparison, PFGE typing resulted in 33 patterns comprised of 9 – 14 fragments in the size range between 550 and 50 kbp [12]. The 38 DDSL patterns consisted of 28 patterns unique to single isolates and ten clusters (two or more isolates with an

identical pattern). PFGE typing performed on 66 isolates (the same 62 isolates plus four additional isolates) resulted in 21 unique patterns and 12 clusters [12].

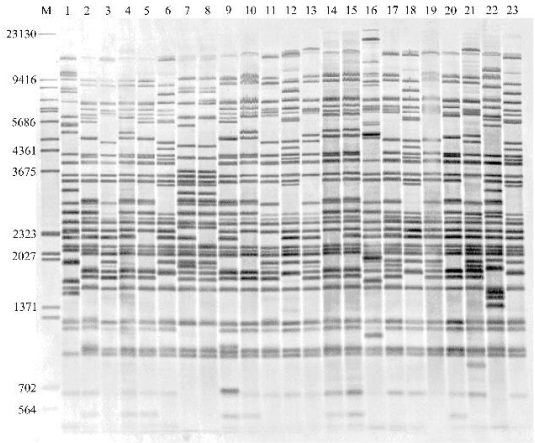

Figure 1 – Representative DDSL fingerprint patterns with the *Cfr*9I + *Hae*II restriction enzyme combination used on 23 methicillin resistant *Staphylococcus aureus* (MRSA) isolates

PFGE typing gave one large cluster representing ten isolates, which could be resolved by DDSL typing into six distinct patterns. One of these patterns included four isolates and the rest were unique. The second largest PFGE cluster included six isolates which could be resolved by DDSL typing into six patterns. The six small (each representing two isolates) PFGE clusters could each be further resolved into two distinct DDSL patterns. On the other hand, the largest DDSL cluster of ten isolates was resolved into seven PFGE patterns, one of which contained four isolates that were identical by both PFGE and DDSL typing. Discriminatory power based on Simpson's index of diversity (http://insilico.ehu.es/DDSL) calculated over all isolates in the study with both methods was 0.96.

Further evaluation of DDSL typing was performed using a set of 15 *S. aureus* isolates obtained from pet animals (Table 1). In a previous report (10) these isolates were characterized by PFGE typing which identified two main PFGE groups (A and B) and four banding patterns (A1 – A2 and B1 – B2). Application of DDSL typing also revealed two groups (C and D) and five banding patterns (C1 – C4 and a unique isolate D). Four isolates belonging to A1 and A2 PFGE patterns (04-3098, 04-3099 and 04-3097, 04-3101, respectively) were resolved by DDSL into patterns C1, C3 and C2, C4, respectively. Interestingly, that DDSL typing, despite its high discriminatory power, did not differentiate between the A1 and B1 PFGE patterns.

Table 1 – Comparison of PFGE and DDSL typing data with 15 *Staphylococcus aureus* (MRSA) strains

Strain I.D.*	PFGE	DDSL
04-3095	A1	C1
04-3097	A2	C2
04-3098	A1	C1
04-3099	A1	C3
04-3100	B2	D
04-3101	A2	C4
04-3103	A1	C3
04-3104	B1	C1
04-3105	A1	C1
04-3106	A1	C1
04-3108	A1	C1
04-3109	B1	C1
04-3110	A1	C1
04-3111	B1	C1
04-3112	A1	C1

* *Isolate identification numbers and PFGE data are from the published paper [10].*

One explanation for the differences between two fingerprinting methods is that they not sampling exactly the same genetic alterations. In that sense, they can be thought of as complementary rather than competing methods. The main polymorphisms sampled by PFGE typing are due to point mutations in the restriction cutting sites for *Sma*I and large genomic rearrangements (phage mobilization, large insertions / deletions etc). The main sources of polymorphism for DDSL typing are point mutations in the restriction cutting sites for the isoschizomer of *Sma*I (*Cfr*9I) and *Hae*II enzymes plus a contribution attributable to small genomic rearrangements (small insertions / deletions). In view of the large genome of bacteria, it is not surprising that different typing methods produce different typing clusters particularly for closely related bacterial strains. For instance, it is well known

that PFGE typing performed on the same set of bacterial isolates with different PFGE enzymes may produce different groupings using standard algorithms for genetic relatedness. Screening a larger number of bands and thus larger number of nucleotide sequences can be advantageous in terms of resolving power. In the current study, DDSL typing with agarose gel analysis was based on 28 – 33 fragments as opposed to the 9 – 14 fragments obtained with PFGE method. Computer analysis of the genome using the DDSL prediction software shows that the total number of fragments after double digestion should be about 50 (twice the number of *Cfr*9I cutting sites in *Staphylococcus aureus* species). Limitations inherit in agarose gel analysis prevent the detection of all DDSL fragments produced as a result of double digestion and it is expected that the application of capillary electrophoresis would bring out the ultimate discrimination potential of DDSL.

An interesting feature of DDSL is that it is not sensitive to incomplete digestion with the *Cfr*9I enzyme. In preliminary tests, progressively lower amounts of this enzyme were included in the reaction mix and the specificity of banding patterns remained constant even though there was a progressively lower signal. In contrast, complete digestion with trimming enzyme was critical for generating reproducible patterns. This observation can be explained by the fact that virtually all long *Cfr*9I fragments are cut with the frequently cutting trimming enzyme, and detected fragments have one end from *Cfr*9I and the other from the trimming enzyme. Loss of some *Cfr*9I cuts due to incomplete digestion does not change the observed fragment sizes which are determined by the trimming enzyme.

Our data suggest that DDSL typing can be successfully applied for short-term epidemiological studies with *Staphylococcus aureus* when the time is critical and in hospital laboratories where specialized equipment for pulsed field electrophoresis is not available. The DDSL typing technique has a potential for application to other bacterial species like *Salmonella* spp. and *Pseudomonas aeruginosa*. Online simulation software was developed which allows performing *in silico* experiments prior to wet ones, and a suggestion tool allows the selection of suitable combination of endonucleases for all up-to-date sequenced prokaryotic species. This tool is of great interest for those researches willing to apply DDSL fingerprinting to species that has not been searched previously with this technique.

Conflict of Interest

None declared.

Конфликт интересов

Не указан.

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