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Terletskiy V.P.[1]*, Novikova O.B.[2], Tyshchenko V.I.[2, 3], Shinkarenko L.A.[3]

[1] Pushkin Leningrad State University, St. Petersburg, Russia;
[2] All Russian Research Veterinary Institute of Poultry Science - Branch of the Federal Scientific Center «All-Russian Research and Technological Poultry Institute», Saint Petersburg-Lomonosov, Russia;
[3] Selective Breeding Genetic Center - Branch of the Federal Scientific Center «All-Russian Research and Technological Poultry Institute», Obiljnoye v., Russia

* Correspodning author (valeriter[at]mail.ru)

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IN-SILICO SELECTION OF ENZYMES FOR STRAIN GENOTYPING OF BACTERIA BELONGING TO *CAMPYLOBACTER* GENUS ISOLATED FROM BIRDS

Research article

Abstract

The article presents modern ideas about the genotyping of pathogens of a bacterial nature as an element in preventive veterinary medicine. Identification of bacterial strains allows one to effectively identify the spread of infection and find pathogen sources in the environment. This knowledge makes it possible to break up the epizootic chain and prevent the further spread of infection. The questions of various methods of genotyping are considered along with their advantages and disadvantages. The results of the genotyping of certain species of microorganisms previously carried out by the authors are presented; the methodological details of the proposed method of bacterial genotyping based on the idea of double digest and selective label of DNA fragments (DDSL) are examined in detail. The use of databases of sequenced bacterial genomes and computer programs that theoretically model the digestion of genomic DNA by restriction endonucleases, allows one to propose this approach for genotyping *Campylobacter* bacteria in the same way as was done earlier when genotyping bacteria of the *Escherichia coli* group, *Salmonella* spp. and *Pseudomonas aeruginosa*. The proposed method is based on the use of two restriction endonucleases at the same time, one of which produces 3'-recessed ends of DNA fragments of a pathogen that can include labeled deoxynucleoside triphosphate, and an enzyme that produces 3'-protruding or blunt ends that are not capable of including labels. Ultimately, 20-50 clearly visible labeled DNA fragments will be detected, the number and distribution of which is characteristic for each bacterial strain. On-line service allows to simulate DNA cleavage in order to select endonucleases which can be recommended for *Campylobacter* genotyping.

Keywords: pathogenic bacteria, *Campylobacter,* preventive veterinary medicine, genotyping, restriction endonucleases.

Терлецкий В.П.*[1], Новикова О.Б.[2], Тыщенко В.И.[2, 3], Шинкаренко Л.А.[3]

[1] Государственное автономное образовательное учреждение высшего образования Ленинградской области «Ленинградский государственный университет им. А.С. Пушкина» (ГАОУ ВО ЛО ЛГУ им. А.С. Пушкина), Санкт-Петербург, Россия;
[2] «Всероссийский научно-исследовательский ветеринарный институт птицеводства» - филиал Федерального государственного бюджетного научного учреждения Федерального научного центра «Всероссийский научно-исследовательский и технологический институт птицеводства», Санкт-Петербург-Ломоносов, Россия;
[3] Северо – Кавказская зональная опытная станция по птицеводству - филиал Федерального государственного бюджетного научного учреждения Федерального научного центра «Всероссийский научно-исследовательский и технологический институт птицеводства», Санкт-Петербург-Ломоносов, Россия

* Корреспондирующий автора (valeriter[at]mail.ru)

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IN-SILICO ПОДБОР ФЕРМЕНТОВ ДЛЯ ГЕНОТИПИРОВАНИЯ ШТАММОВ БАКТЕРИЙ РОДА КАМПИЛОБАКТЕР, ВЫДЕЛЕННЫХ У ПТИЦ

Научная статья

Аннотация

В статье изложены современные представления о генотипировании патогенов бактериальной природы как элемента в превентивной ветеринарии. Идентификация бактериальных штаммов позволяет эффективно выявлять пути распространения инфекции и находить источники патогена во внешней среде. Эти знания дают возможность прервать эпизоотическую цепь и не допустить распространения инфекции. Рассматриваются вопросы различных методов генотипирования. их преимущества и недостатки. Представлены ранее полученные авторами результаты по генотипированию отдельных видов микроорганизмов, подробно рассмотрены методические детали предлагаемого метода генотипирования бактерий, основанного на идее двойного расщепления и избирательного мечения фрагментов ДНК (ДРИМ). Использование баз данных секвенированных геномов бактерий и компьютерных программ, позволяющих теоретически моделировать расщепление геномной ДНК эндонуклеазами рестрикции, позволяет предложить подход к генотипированию бактерий рода *Campylobacter* по аналогии, как это было сделано ранее при генотипировании бактерий группы кишечной палочки, сальмонелл и псевдомонад. Предлагаемый метод основан на использовании одновременно двух эндонуклеаз рестрикции, одна из которых производит 3'-усеченные концы фрагментов ДНК патогена, способные включать меченый дезоксинуклеозидтрифосфат, и фермента, производящего 3'-выступающие либо тупые концы, не способные к включению метки. В конечном итоге будет детектироваться 20-50 четко различимых меченых фрагментов ДНК, число и распределение которых характерно для каждого бактериального штамма. Онлайн-сервис позволяет смоделировать расщепление для быстрого выбора эндонуклеаз, которые можно рекомендовать для генотипирования бактерий рода *Campylobacter*.

Ключевые слова: патогенные бактерии, *Campylobacter,* профилактическая ветеринария, генотипирование, эндонуклеазы рестрикции.

1. Introduction

Molecular genetic confirmation of the spread of infection and the identification of pathogen sources are relevant issues in the system of preventive measures aimed at combating infectious diseases in humans and animals. Diagnosis of the disease by PCR analysis makes it possible only to indicate the biological species of the pathogen; methods are needed to more accurately identify the genetic variants of the microorganism within the species. A fast and accurate method for identifying bacterial strains allows one to scientifically justify effective preventive measures. Campylobacteriosis is a problem in both human and veterinary medicine. The natural reservoir of *Campylobacter* bacteria is various species of animals and birds [1].

Currently, there are a large number of methods for genotyping bacteria, each of which has advantages and disadvantages [2]. Methods for detecting genetic variations in the nucleotide sequence in individual bacterial genes by sequencing (MLST) may not give the necessary resolution in dividing a group of isolates into clusters [6]. Many researchers [6; 7] prefer the method of genotyping, based on the separation of DNA fragments of the pathogen by pulse-field gel electrophoresis (PFGE). In recent years, alternative high-tech genotyping techniques using Raman [9] and mass spectroscopy [8] approaches has been applied. The method of detecting polymorphic DNA repeats using the polymerase chain reaction MLVA [10], in combination with PFGE and MLST to increase discriminatory power has been verified [4]. In general, the PFGE method has a high discriminatory ability; however, it is time-consuming and quite laborious to complete all its experimental stages.

Previously, we developed a method for genotyping microorganisms based on the idea of double digest selective label (DDSL), which demonstrated the highest level of discrimination (D> 0.96) for several species of microorganisms. Existing databases of sequenced genomes of a number of pathogenic bacteria, as well as available computer programs for *in-silico* modeling of genomic DNA digestion with restriction endonucleases [5], allow preliminary selection of candidate enzymes for genotyping. *In-silico* analysis provides necessary data to select the optimal combination of restriction enzymes for each biological species of the pathogen. In particular, for *E. coli* and *Salmonella* spp., the optimal choice is to use a combination of *Xba*I/*Pst*I enzymes, for *Pseudomonad aeruginosa* - *Spe*I/*Stu*I. The genomes of many species of pathogenic *Campylobacter* are sequenced and it is possible, using the *in-silico* program (http://insilico.ehu.es/), to select a pair of restriction endonucleases for genotyping by the DDSL method. This method [3] is based on the simultaneous digestion of the genomic DNA of a microorganism by two restriction endonucleases and selective labeling of individual DNA fragments. The first restriction endonuclease, which has 10–40 recognition and cleavage sites in the genome, forms a limited number of fragments with so-called "sticky ends" (3'-recessed ends) that can incorporate a label (biotinylated deoxycytosynthosphate - Bio-dCTP) upon enzymatic hydrolysis of DNA. DNA fragments formed by a second restriction endonuclease having about 1000 recognition sites give only the blunt or 3'-protruding ends of the fragments that cannot incorporate the label. It is known that the genomes of most pathogenic bacteria consist of 2,000-6,000 thousand base pairs of DNA. Given the frequency of genome cleavage by the second enzyme, the resulting average fragment size will be 2000-6000 base pairs, which is optimal from the point of view of separation of these fragments in a conventional agarose gel. Such selectivity of labeling allows reducing the number of DNA fragments from many thousands to several tens and to resolve them during electrophoresis step and subsequently detect this limited number of fragments on the filter.

The aim of the work is *in-silico* selection of the optimal pair of restriction endonucleases for use in the genotyping of *Campylobacter* by the DDSL method and to perform DDSL genotyping with several bacterial species.

2. Methods

The first stage of work is the cultivation of a microorganism in a suitable medium in order to obtain a pure culture (isolate). Preliminary experiments have shown that for gram-negative bacteria of the *Campylobacter* genus, the standard DNA extraction procedure using the phenol-chloroform extraction step is suitable. The overnight culture was centrifuged in 1.5 ml Eppendorf tubes for 5 minutes at 8000g. The bacterial cell pellet is then used to isolate DNA. DNA is dissolved in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Methodically, the DDSL protocol is very simple: 15 µl of water, 2 µl of restriction endonuclease buffer, 2 µl of DNA and 1 µl of the enzyme mixture were added to the tube. The enzyme mixture is made by mixing two restriction enzymes, Taq polymerase and bio-dCTP. After the completion of the DDSL reaction, the double digested and labeled products are added to the wells of a standard 0.8% agarose gel. Electrophoresis is carried out for 16 hours (overnight) at a voltage of 60 volts; the length of the gel is 25 cm. At the end of electrophoresis, the DNA fragments are transferred to a nylon filter. For this we use a vacuum transfer apparatus. The procedure lasts 30 minutes at a vacuum level of 30-40 MPa in distilled water. Fixation of transferred DNA fragments on the filter is achieved by irradiating the filter with ultraviolet for 4 minutes in a transilluminator. Biotinylated DNA fragments are detected on the filter using standard reagents for the detection of alkaline phosphatases (NBT and BCIP dyes). We use the streptavidin-alkaline phosphatase conjugate to bind it with biotinylated fragments [5].

The genomes of many species of pathogenic *Campylobacter* are sequenced and it is possible, using the *in-silico* program (http://insilico.ehu.es/), to select a pair of restriction endonucleases for genotyping by the DDSL method. For *Campylobacter* species various combinations of restriction enzymes can be suggested. Promising ones are *Avr*II/*Rsa*I, *Avr*II/*Hha*I, *Pau*I/*Bsu*RI, and *SalI*/*Bsu*rI.

3. Results and discussion

The result of genotyping by the DDSL method is a group of DNA fragments in the form of colored bands on the filter, the distribution of which is specific for each strain. Previous experiments on the genotyping of isolates *Clostridium difficile*, *Pseudomonas aeruginosa*, and *Salmonella enterica* proved both the effectiveness of the method and the predictability of the number of detected DNA fragments depending on the number of restriction endonuclease cleavage sites. Typically, the number of DNA fragments on the filter is from 20 to 50, and they are distributed along the length of the electrophoretic lane so that their position is easy to identify (Figure 1).

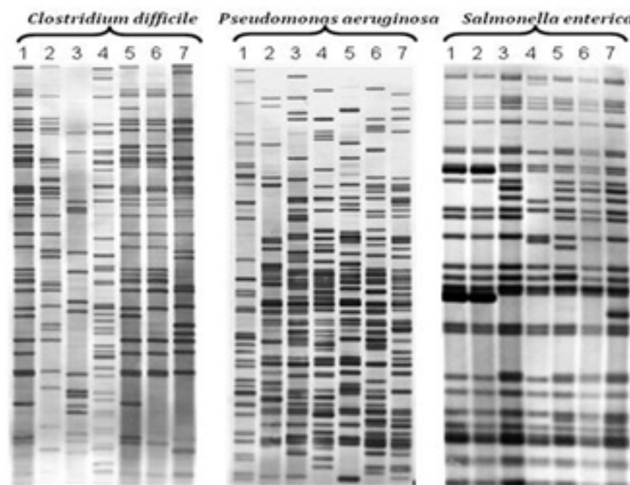

Figure 1 – Genotyping three pathogenic bacteria - *C. difficile*, *P. aeruginosa* and *S. enterica* by DDSL technique

When all fragments in two bacterial isolates under comparison have the same position on the filter, we conclude about their genetic identity, i.e. isolates probably have one source or they are clones of the same strain. In this case, we can talk about the transmission of infection from one animal to another. It is often possible to detect also closely related strains that have most DNA fragments in common. As a rule, epizootologically non-related strains (different regions, years) have completely different genetic profiles. Genotyping of *Escherichia coli* isolates isolated from sick and dead chickens of various poultry farms in the country allowed us to prove the transmission of infection between individual poultry house and between individuals within the same facility.

Using databases of sequenced bacterial genomes with a program for simulating genome cleavage by restriction endonucleases (*http://insilico.ehu.es/*) allowed the preliminary selection of enzymes for genotyping of *Campylobacter* bacterial isolates (tables 1 and 2). In the selection, the following enzyme properties were taken into account: the number of recognition and cleavage sites in the genome of *Campylobacter*, the sensitivity to DNA methylation, the maximum value of enzyme specificity, and the ability to show maximum activity in various reaction buffers.

Analysis of all combinations of enzymes allowed us to recommend the following optimal combinations of enzymes and buffers:

1. *Avr*II/*Rsa*I, *Avr*II/*Hha*I, буфер Tango™, activity of both enzymes in this buffer is - 100%;
2. *Pau*I/*Bsu*RI, буфер R, activity of both enzymes in this buffer - 100%
3. *Sal*I/*Bsu*rI, буфер O, activity of *Sal*I - 100%, activity of *Bsu*rI - 50-100%

Due to the fact that the molecular hybridization reaction in the DDSL will not be carried out, there is no need to denature and neutralize DNA in the gel. Therefore, the steps of denaturation and neutralization necessary for the classical Southern transfer technique are skipped in our case. This greatly speeds up and simplifies the procedure.

Table 1 – Selected restriction endonucleases forming 3'-recessed ends for genotyping of *Campylobacter* using the DDSL method and their main properties

Properties / Enzyme	Recognition and cleavage site	Numer of sites in *C. jejuni* 4031	Number of sites in *C. coli* 76339	Optimal buffer	Specificity*
*Cfr*9I	C↓CCGGG	11	13	Cfr9I	160
*Sal*I	G↓TCGAC	6	10	O	80
*Avr*II	C↓CTAGG	62	76	Tango™	160
*Pau*I	G↓CGCGC	19	48	R	160
*Eco*52I	C↓GGCCG	13	11	Eco52I	160

* *Specificity of the enzyme is expressed as a maximum excess of the enzyme, which still does not cause non-specific DNA hydrolysis*

Table 2 – Selected restriction endonucleases forming 3'-protruding or blunt ends for genotyping of *Campylobacter* using the DDSL method and their main properties

Properties / Enzyme	Recognition and cleavage site	Numer of sites in *C. jejuni* 4031	Numer of sites in *C. coli* 76339	Optimal buffer	Specificity
*Rsa*I	GT↓AC	1297	1568	Tango™	160
*Hha*I	GCG↓C	1176	1791	Tango™	160
*Bsu*RI	GG↓CC	644	571	R	160
*Ssp*I	AAT↓ATT	1936	1550	G	15

Thus, all three mentioned above combinations of enzymes can be recommended for experimental confirmation of their effectiveness in genotyping by the DDSL method. However, it is necessary to take into account the individual specific features of their use. So, the first nucleotide to be included in the 3'-recessed DNA fragments formed by the *Sal*I enzyme will be deoxythymidine triphosphate (dTTP), therefore, in addition to the bio-dTCP label, this deoxynucleoside triphosphate should be included in the reaction mix to fill the first position in recessed ends. In other enzyme combinations this is not necessary.

Comparing the potential usefullness of the *Avr*II/*Rsa*I and *Avr*II/*Hha*I combinations, it is worthwhile to mention the negative intrinsic property of the *Rsa*I enzyme, which is that the enzyme has a reduced cleavage activity at methylated CpG sites in the genome, which may result in incomplete digestion of genomic DNA sites and subsequent difficulties in interpreting the distribution of detected fragments (band smearing). The *Hha*I enzyme does not cleave methylated sites of CpG, which may be an advantage. *Avr*II cleaves DNA regardless of its methylation, as opposed to the *Pau*I enzyme, which does not recognize methylated CpG sites and can be used in combination with the *Bsu*RI enzyme, which is not sensitive to methylation. The *Sal*I enzyme does not cleave methylated CpG sites and, considering that the theoretical number of cleavage sites in the genomes of the two *Campylobacter* species (*C. jejuni* and *C. coli*) is only 6 and 10 (Table 1), this enzyme should not be considered optimal for genotyping, moreover, additional dTTP is required in this case for the reaction.

4. Conclusion

Taking into account mentioned above data, we can propose the *Avr*II/*Hha*I as the enzymes of choice for genotyping isolates of *Camplylobacter* bacteria. Both enzymes work in the same buffer Tango™ with 100% activity; very interesting is the property of *Hha*I not to cleave methylated CpG sites. In this case, it is possible to detect differences between strains with identical DNA sequences, but having differences in methylation patterns. It means that the DDSL genotyping can reveal inter-strain differences where conventional sequencing will not discriminate compared strains.

Conflict of Interest

None declared.

Конфликт интересов

Не указан.

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