ANIMAL HUSBANDRY

DOI: https:/doi.org/10.23649/jae.2019.4.12.14

Atsaeva M.M.[1] *, Shakhbiev I.Kh.[2], Tamriev A.I.[3], Kiskaev R.H.[4]

[1, 2, 3, 4] Chechen State University, Grozny, Russia

* Correspodning author (acaeva-mm[at]mail.ru)

Received: 14.11.2019; Accepted: 30.11.2019; Published: 16.12.2019

EFFICIENT TECHNIQUE FOR FAST IDENTIFICATION OF *SALMONELLA* SPP. AND *PROTEUS* BACTERIAL STRAINS

Discovery notes

Abstract

A method for the rapid genotyping of microorganisms using the double digest and selective labeling (DDSL method) was developed to identify strains of pathogenic serotypes of *Salmonella* spp. and *Proteus*. Bacterial isolates were identified on species level and grown in liquid media from collected samples (tissues and bird droppings in different years and from different places). The results of the genotyping of these isolates are consistent with epizootological data, so this method can be recommended for practical use in identifying the routes of infection transmission and localizing the source of pathogens in environment.

Keywords: *Salmonella* spp., *Proteus*, bacteria, isolates, genotyping technique, pathogens.

Ацаева М.М.[1], Шахбиев И.Х.*[2], Тамриев А.И.[3], Кискаев Р.Х.[4]

[1, 2, 3, 4] Федеральное государственное образовательное учреждение «Чеченский государственный университет», Грозный, Россия

* Корреспондирующий автора (acaeva-mm[at]mail.ru)

Получена: 14.11.2019; Доработана: 30.11.2019; Опубликована: 16.12.2019

ЭФФЕКТИВНЫЙ МЕТОД БЫСТРОЙ ИДЕНТИФИКАЦИИ БАКТЕРИАЛЬНЫХ ШТАММОВ САЛЬМОНЕЛЛ И ПРОТЕЯ

Исследовательские заметки

Аннотация

Разработан метод быстрого генотипирования микроорганизмов с использованием метода двойного расщепления и избирательного мечения (метод ДРИМ) для идентификации штаммов патогенных штаммов сальмонелл и протея. Бактериальные изоляты идентифицировали на видовом уровне и выращивали в жидкой питательной среде из собранных образцов (тканей и помета птиц в разные годы и из разных мест). Результаты генотипирования штаммов соответствуют эпизоотологическим данным, таким образом, этот метод можно рекомендовать для применения в практике при выяснении способов распространения инфекций и нахождения резервуара патогенов в окружающей среде.

Ключевые слова: сальмонелла, протей, бактерии, изоляты, метод генотипирования, патогены.

1. Introduction

The rapid identification of pathogenic bacterial strains is currently of particular relevance [1], [2]. This is due to the circulation of pathogens in the environment and periodic endemic outbreaks of disease [3]. In poultry farming, infectious diseases, primarily salmonellosis, pose a particular threat to poultry industry [4], since in poultry farms birds iare kept in conditions facilitating the transmission of microorganisms between individuals (crowding, dust in the facilities, etc.). For reliable identification and certification of bacterial strains, the use of modern methods of genotyping is necessary [5]. Genotyping allows one to assign a molecular genetic "barcode" to each strain, in order to trace transmission routes and identify sources of infection. If two isolates collected from different places have the same genotype, we can speak with a high degree of confidence about epizootic contact between individuals. In addition, strain certification is important when storing collections of strains, including vaccines, in the laboratory. There are many methods for genotyping animals and microorganisms. Currently, it has been unequivocally proven that methods based on genomic DNA polymorphism (genotyping) are the most sensitive and reproducible [6], [7].

To date, the most accurate gel-based method of genotyping is the DDSL method, which was first developed for clinical isolates of pathogenic microorganisms - *Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella* spp. [8], [9], [10]. The result of genotyping by the DDSL method is a group of DNA fragments visualized as distinctive bands on the filter, the distribution of which is specific for each strain. The accuracy of identification of strains calculated by the discrimination index [11] exceeds the accuracy of the current "gold standard" gel-based techniques for genotyping like pulse-field gel electrophoresis and reaches 0.98 for *Pseudomonads* and 0.96 for *Salmonella* [8], [9].

The purpose of this work was to elucidate the correspondence of the genotyping data by the DDSL method and known epizootological data (time, place of sampling) on the *Salmonella* and *Proteus* isolates.

2. Material and methods

The study object was 9 bacterial isolates of *Salmonella* (S. enteritidis, S. gallinarum, and S. typhimuriumserovars) and 8 isolates of *Proteus* (P. vulgaris and P. mirabilis) isolated from tissues or bird droppings . Genomic DNA was isolated in the traditional way using phenol-chloroform extraction. The resulting DNA was washed with 70% ethanol, dried and dissolved in TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0).

The DDSL method is based on the simultaneous digestion of the genomic DNA of a microorganism by two restriction endonucleases and the selective labeling of individual DNA fragments. Due to the fact that in many types of microorganisms the genome is currently completely sequenced, i.e. the sequence of nucleotides in genomic DNA is determined, it is possible to theoretically predict the number of DNA fragments obtained by digestion of each of the restriction enzymes, as well as after simultaneous double digestion by two restriction enzymes. To do this, we use the program available on the Internet (http://insilico.ehu.es/DDSL). The program was developed by researchers from Spain [12], who also participated in the development of the DDSL method for clinically important types of pathogenic bacteria as part of joint NATO-Russia grants. An *in-silico* search revealed that the best candidate digesting restriction enzyme for *Proteus* is *Sgs*I, which has a GG ↓ CGCGCC recognition and cleavage site. This enzyme has several tens of cleavage sites in the *Proteus* genome and produces "sticky" ends that are labeled with biotinylated deoxycytosine triphosphate (Bio-dCTP) using Taq polymerase [8], [9], [10]. The resulting DNA fragments cannot be separated on a conventional agarose gel, as they are too large. Therefore, the second "trimming" restriction enzyme E*co*32I was introduced into the reaction, having about a thousand cleavage sites and recognizing the GAT↓ATC sequence. As a result of this double digestion, the size of the DNA fragments is optimal for separation on an agarose gel. Thus, a limited number of labeled DNA fragments are present in the reaction mixture that can be separated and subsequently visualized. The result of the DDSL reaction is that the most generated DNA fragments have either blunt or 3-prime protruding ends that cannot include Bio-dCTP, the smaller part (several tens) of fragments will have incorporated Bio-dCTP tags.

Genotyping of *Salmonella* was carried out in a similar way, except for the selection of other restriction enzymes: first restriction enzyme is XbaI and the "trimming"enzyme – P*st*I which produces 3-prime protruding fragment ends uncapable of incorporating Bio-dCTP tags.

Technically, double digestion and selective labeling (DDSL) includes adding 15 µl of water, 2 µl of 10-fold corresponding buffer (Thermo Fishcher Sci ™), 2 µl of the isolated genomic DNA and 1 µl of the enzyme mixture (two restriction enzymes, Taq polymerase and Bio-dCTP label). The mixture was incubated for 2-3 hours at 37°C. Electrophoresis was performed on a 0.8% agarose in 20 cm gel for 16 hours (overnight). The transfer of the separated DNA fragments to a nylon filter was carried out immediately after electrophoresis in distilled water on a vacuum device (denaturation and neutralization of DNA as in traditional Southern blotting is not required). Detection of DNA fragments on the filter was carried out using the standard color chemical reaction based on the detection of alkaline phosphatise (two dyes are used: NBT and BCIP) [10].

3. Results and discussion

The DDSL method we developed allows one to simultaneously identify about 35 DNA fragments, which is a record indicator for multilocus genotyping methods (RAPD - 5-10 fragments, pulsed-field gel electrophoresis - 15-20 fragments). The more DNA fragments are taken into account in the analysis, the more sensitive this method becomes. Genotyping of bacterial genomic DNA revealed the identity of three isolates of Salmonella gallinarum (S. gallinarum) - 2Sg, 3Sg and 4Sg and Salmonella enteritidis (S. enteritidis) - 1Se and 3Se (Table 1). Among 8 *Proteus* isolates, two were identical (6 and 7).

Table 1 – Genotyping of isolates of Salmonella spp. and Proteus by the DRIM method

Salmonella spp. (*Xba*I/*Pst*I)		*Proteus vulgaris/mirabilis* (*Sgs*I/*Eco*32I)	
№ изолята*	№ генотипа	№ изолята**	№ генотипа
1St	1	1Pv	1
1Sg	2	2Pm	2
2Sg, 3Sg, 4Sg	3	5Pv	3
1Se, 3Se	4	6Pv, 7Pv	4
4Se	5	8Pv	5
5Se	6	11Pm	6
		12Pv	7

** St – S. typhimurium, Sg - S. gallinarum, Se - S. enteritidis*

***Pv – P. vulgaris, Pm - P. mirabilis*

The DDSL genotyping data are in good agreement with epizootological data (location, time of sampling). In particular, S. gallinarum isolates were grown from tissues of sick chickens that were in close contact in one farm (Uzbekistan). The identity of the *Salmonella* genotype indicates that the chickens are infected from each other by the same strain of the pathogen. Bacterial cultures of S. gallinarum were grown from different organs of affected chickens: 2Sg - heart, February 2009, 3Sg - liver, February 2009, 4Sg - egg follicles, February 2009. Genotyping of Salmonella enteritis revealed the identity of isolates isolated from the broiler in Belgorod region (2012) and individuals at the farm in Leningrad region (2006). This indicates a possible transmission of the pathogen between these farms through possible contact.

Proteus isolates according to genotyping data were represented by genetically distinct strains. The exception is isolates 6 and 7. These two bacteria were grown from quail litter in one poultry farm in the Leningrad region in 2010 and 2011 and its no surpize that they were identical. All other samples were taken from other places.

The next stage of the work was devoted to the quantitative assessment of differences between bacterial strains. To achieve this goal, the number of differing DNA fragments in the DDSL banding patterns were calculated (Table 2). Strain 1S.t. (Salmonella typhimurium) was significantly different from other strains. At the same time, strains of Salmonella gallinarum and Salmonella enteritidis differed from each other by smaller number of fragments.

Table 2 – Differences between the isolates of Salmonella typhimurium (isolate 1St), Salmonella gallinarum (isolates 1Sg-4Sg) and Salmonella enteritidis (isolates 1Se, 3Se-5Se) in the number of different fragments revealed by DDSL genotyping technique

	1St	1Sg	2Sg	3Sg	4Sg	1Se	3Se	4Se	5Se
1St	0	36	34	34	34	33	33	33	41
1Sg		0	13	13	13	14	14	14	35
2Sg			0	0	0	14	14	14	32
3Sg				0	0	14	14	14	32
4Sg					0	14	14	14	32
1Se						0	0	0	22
3Se							0	0	22
4Se								0	22
5Se									0

A similar calculation of the number of common and different DNA fragments in *Proteus* isolates revealed the extremely high genetic distance of isolate 11 (*P. mirabilis*) from the remaining isolates (*P. vulgaris*), the fact that can be anticipated. The number of distinct DNA fragments ranged from 40 to 47. The *P.vulgaris* isolates differed from each other by 1–11 fragments. Isolates 6 and 7 did not differ in DNA fragments, i.e. they were genetically identical (table. 3).

Table 3 – Differences between isolates of *Proteus vulgaris* (Pv) and *Proteus mirabilis* (Pm) in the number of different DNA fragments

№ изолята	1Pv	5Pv	6Pv	7Pv	8Pv	11Pm	12Pv
1Pv	0	5	1	1	8	40	10
5Pv		0	3	3	7	45	10
6Pv			0	0	11	40	11
7Pv				0	11	40	11
8Pv					0	45	7
11Pm						0	47
12Pv							0

These data allow us to build a tree of genetic relationships (UPMGA algorithm) between *Proteus* isolates (Figure 1).

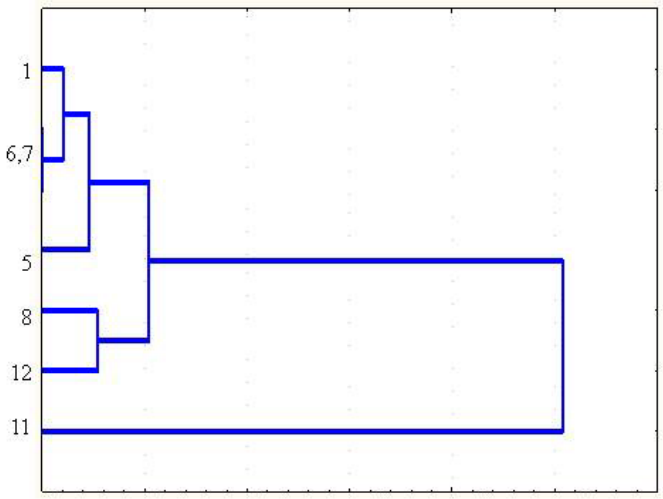

Figure 1 – Dendrogram showing genetic distances between Proteus isolates

4. Conclusion

Thus, the DDSL method allows one to identify individual strains and groups of closely related strains of microorganisms of the genera *Salmonella* and *Proteus* and to judge about their genetic divergence. The method can be effectively used when finding the source of infection and identifying the routes of bacterial pathogen spread in the environment and between individuals.

Conflict of Interest

None declared.

Конфликт интересов

Не указан.

Funding

This work was supported by the RFBR grant on application 18-316-00051 mol_a "Development and use of express method for identification of pathogens of bacterial nature in solving problems of molecular epizootology (identification of the source of infection, identification of pathways)"

Финансирование

Работа поддержана грантом РФФИ по проекту 18-316-00051 мол_а «Разработка и использование экспресс-метода идентификации патогенов бактериальной природы в решении вопросов молекулярной эпизоотологии (идентификация источника инфекции, выявление путей распространения)».

References

1. Жебрун А.Б. Генотипирование и субтипирование патогенных микроорганизмов в развитии технологий эпидемиологического надзора / А.Б. Жебрун, С.А. Мукомолов, О.В. Нарвская // Медицинский академический журнал. – 2009. – №4. – С.59–67.
2. Тапальский Д.В. Фенотипическое и молекулярно-генетическое типирование сальмонелл: реалии и перспективы / Д.В. Тапальский, В.А. Осипов, С.В. Жаворонок // Журнал микробиологии, эпидемиологии и иммунобиологии. – 2005. – №6. – С.88–93.
3. Добрина М. Н. Нужен постоянный контроль сальмонеллеза / М. Н. Добрина // Животноводство России. – 2011. – №3. – С.11-13.
4. Борисенкова А. Н. Эффективность препаратов разных классов для контроля сальмонеллы энтеритидис / А. Н. Борисенкова, О. Б. Новикова, М. Н. Байбарак, А. В. Варюхин // Материалы XVI конференции «Достижения в современном птицеводстве: исследования и инновации». – Сергиев Посад. – 2009. – С.344–347.
5. Ботина С.Г. Молекулярно-генетическая идентификация, ДНК-генотипирование и паспортизация молочнокислых бактерий рода Lactobacillus / С.Г. Ботина // Доклады на V Съезде генетиков и селекционеров. – Москва. – 2009. – С.49.
6. Lukinmaa S. Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens / S. Lukinmaa, U-M. Nakari, M. Eklund, A. Siitonen // APMIS. – 2004. – V.112. – P.908–929.
7. Яковлев А.Ф. Влияние гена гормона роста на хозяйственные признаки птицы / А.Ф. Яковлев, В.П. Терлецкий, Э.А. Сексте, Л.И. Тучемский, Ж.В. Емануйлова // Птицеводство. – 2013. – № 1. – С. 2–4.
8. Terletskiy V. Application and evaluation of double digest selective label (DDSL) typing technique for Pseudomonas aeruginosa hospital isolates / V. Terletskiy, G. Kuhn, P. Francioli, D. Blanc // Journal of Microbiological Methods. –2008. – V.72. – P. 283–287.
9. Terletskiy V. Validation of Double Digest Selective Label database for sequenced prokaryotic genomes / V. Terletskiy, V. Tyshchenko, I. Martinez-Ballesteros, J. Bikandi, J. Garaizar // Bioinformatics. – 2010. – V. 26. – P.417–418.
10. Terletski V.P. Subtracted restriction fingerprinting – a tool for bacterial genome typing / V.P. Terletski, S. Schwarz, J. Carnwath, H. Niemann // BioTechniques. – 2003. – V. 34. – P. 304–313.

11. Hunter P.R. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity / P.R. Hunter, M.A. Gaston // Journal of Clinical Microbiology. – 1988. – V.26. – P.2465–2466.

12. Bikandi J. In silico analysis of complete bacterial genomes: PCR, AFLP-PCR, and endonuclease restriction / J. Bikandi, R. San Millan, A. Rementeria, J. Garaizar // Bioinformatics. – 2004. – V.22. – P. 798–799.

References in English

1. Zhebrun A.B. Genotipirovanie i subtipirovanie patogenny`x mikroorganizmov v razvitii texnologij e`pidemiologicheskogo nadzora [Genotyping and subtyping of pathogenic microorganisms in the development of epidemiological surveillance technologies] / A.B. Zhebrun, S.A. Mukomolov, O.V. Narvskaya // Medicinskij akademicheskij zhurnal. – 2009. – №4. – P.59–67. [in Russian]

2. Tapal`skij D.V. Fenotipicheskoe i molekulyarno-geneticheskoe tipirovanie sal`monell: realii i perspektivy` [Phenotypic and molecular genetic typing of Salmonella: realities and prospects] / D.V. Tapal`skij, V.A. Osipov, S.V. Zhavoronok // Zhurnal mikrobiologii, e`pidemiologii i immunobiologii. – 2005. – №6. – P.88–93. [in Russian]

3. Dobrina M. N. Nuzhen postoyanny`j kontrol` sal`monelleza [Need constant monitoring of salmonellosis] / M. N. Dobrina // Zhivotnovodstvo Rossii. – 2011. – №3. – P.11-13. [in Russian]

4. Borisenkova A.N. E`ffektivnost` preparatov razny`x klassov dlya kontrolya sal`monelly` e`nteritidis [The effectiveness of drugs of different classes for the control of salmonella enteritis] / A. N. Borisenkova, O. B. Novikova, M. N. Bajbarak, A. V. Varyuxin // Materialy` XVI konferencii «Dostizheniya v sovremennom pticevodstve: issledovaniya i innovacii». – Sergiev Posad. – 2009. – P.344–347. [in Russian]

5. Botina S.G. Molekulyarno-geneticheskaya identifikaciya, DNK-genotipirovanie i pasportizaciya molochnokisly`x bakterij roda Lactobacillus [Molecular genetic identification, DNA genotyping and certification of lactic acid bacteria of the genus Lactobacillus] / S.G. Botina // Doklady` na V S``ezde genetikov i selekcionerov. – Moscow. – 2009. – P.49. [in Russian]

6. Lukinmaa S. Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens / S. Lukinmaa, U-M. Nakari, M. Eklund, A. Siitonen // APMIS. – 2004. – V.112. – P.908–929.

7. Yakovlev A.F.V. Vliyanie gena gormona rosta na xozyajstvenny`e priznaki pticy [Effect of growth hormone gene on poultry economic traits] / A.F. Yakovlev, V.P. Terleczkij, E`.A. Sekste, L.I. Tuchemskij, Zh.V. Emanujlova // Pticevodstvo. – 2013. – № 1. – P. 2–4. [in Russian]

8. Terletskiy V. Application and evaluation of double digest selective label (DDSL) typing technique for Pseudomonas aeruginosa hospital isolates / V. Terletskiy, G. Kuhn, P. Francioli, D. Blanc // Journal of Microbiological Methods. –2008. – V.72. – P. 283–287.

9. Terletskiy V. Validation of Double Digest Selective Label database for sequenced prokaryotic genomes / V. Terletskiy, V. Tyshchenko, I. Martinez-Ballesteros, J.Bikandi, J.Garaizar // Bioinformatics. – 2010. – V. 26. – P.417–418.

10. Terletski V.P. Subtracted restriction fingerprinting – a tool for bacterial genome typing / V.P. Terletski, S. Schwarz, J. Carnwath, H. Niemann // BioTechniques. – 2003. – V. 34. – P. 304–313.

11. Hunter P.R. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity / P.R. Hunter, M.A. Gaston // Journal of Clinical Microbiology. – 1988. – V.26. – P.2465–2466.

12. Bikandi J. In silico analysis of complete bacterial genomes: PCR, AFLP-PCR, and endonuclease restriction / J. Bikandi, R. San Millan, A. Rementeria, J. Garaizar // Bioinformatics. – 2004. – V.22. – P. 798–799.