Laboratory Exercise

Random Amplified Polymorphic DNA PCR in the Teaching of Molecular Epidemiology[S]

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Abstract

In this article, we describe a basic practical laboratory designed for fifth-year undergraduate students of Microbiology as part of the Epidemiology course. This practice provides the students with the tools for molecular epidemiological analysis of pathogenic microorganisms using a rapid and simple PCR technique. The aim of this work was to assay RAPD-PCR technique in order to infer possible epidemiological relationships. The activity gives students an appreciation of the value of applying a simple molecular biological method as RAPD-PCR to a discipline-specific question. It comprises a three-session laboratory module to genetically assay DNAs from strains isolated from a food outbreak.

Keywords: *RAPD-PCR; molecular epidemiology; laboratory exercises*

Introduction

Advances in molecular biology techniques are needed for the integration of current molecular biology methods to be taught in undergraduate laboratory courses. There are several techniques for molecular epidemiology studies. The teaching of molecular techniques is difficult because of limited resources such as funding, faculty expertise and levels of student experience.

The Epidemiology Course for Microbiology career at Universidad Nacional de Río Cuarto in Argentina, corresponds to the 5th year of this career. Since the process of teaching and learning should be dynamic and continuous in order to incorporate new concepts related to what was previously learnt, we will try to establish a dialogue with students in the pursuit of knowledge developed in previous courses or by their own experience in their lives. This methodology revalues the role of prior knowledge in the teaching learning. The theoretical and practical training acquired in this course helps students in the use of epidemiological tools in the professional future, both in the university and in the nonuniversity areas.

In this article, we described a laboratory practice that includes training in a molecular epidemiologic technique using RAPD-PCR. The students need to have previous theoretical knowledge about basic microbiology, bacteriology, bacterial genetics, and molecular biology from previous courses taken in the career.

Staphylococcus aureus is a bacterium involved in a variety of diseases in humans and animals. This bacterium is an important foodborne pathogen involved in a variety of invasive diseases. Of particular relevance is the ability of some strains to produce heat stable enterotoxins that cause staphylococcal food poisoning, which ranks as one of the most prevalent worldwide cause of gastroenteritis. This pathogen can gain access to milk either by direct excretion from udders with clinical and subclinical staphylococcal mastitis or by environmental contamination during the handling and processing of raw milk [1].

A rational and effective strategy to control diseases caused by *S. aureus* may need to be directed against clones that commonly cause infections [2]. Clonally related organisms are members of the same species that share virulence factors, biochemical traits, and also genomic characteristics.

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In order to know if two strains are clonally related it is necessary to typify the strains. The process consists on the analysis of bacterial polymorphisms. It is important in Epidemiology to recognize outbreaks of infection, to detect the cross-transmission of pathogens, to determine the source of infection, to recognize particularly virulent strains of organisms and to monitor vaccine programs [2].

Several molecular typing methods have been described in order to obtain an accurate and fast characterization of *S. aureus* isolates such as coagulase (*coa*) or protein A (*spa*) restriction fragment length polymorphism (RFLP), multiple-locus variable-number tandem-repeat (MLVA), pulse field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and amplified fragment length polymorphism (AFLP). Polymerase chain reaction (PCR) technology was developed during the 1970s and 80s [3]. Then, a range of PCR-related methods have been developed. Random amplification of polymorphic DNA (RAPD PCR) has been applied extensively to distinguish different isolates of *S. aureus* [4, 5]. RAPD-PCR uses short oligonucleotide primers of arbitrary sequence and annealing conditions that favor nonspecific template binding [6]. RAPD primers amplify sequences that are repetitive or unique, and amplification product occurs wherever the primers bind in a converging orientation. The resulting amplicons can be used as a genetic fingerprint. As RAPD-PCR has widespread applications in microbiological research, it was decided to introduce it to the undergraduate Epidemiology Laboratory course.

This practical laboratory provides the students with not only the tools that enable molecular epidemiologic analysis of pathogenic microorganisms by a simple method as RAPD-PCR but also the knowledge to recognize the importance of molecular methods in microbiology. Our experience in developing a RAPD-PCR technique indicates that it can be a straightforward and cost effective addition to the undergraduate microbiology laboratory curriculum.

The aim of this practice is to genotypically characterize *S. aureus* strains related to a food outbreak by RAPD-PCR. Furthermore, identification of genetic fingerprints leads to infer possible epidemiologic links and determine the source of infection.

Experimental Procedures

Preliminary

The Epidemiology Course is given in the third trimester of the fifth year of the career of Microbiology. Three-session laboratory module of 3 hr was designed to develop this practice. The students, about 10, were divided into 2 groups. Each group assayed the same genomic DNAs from *S. aureus* strains collected from a food outbreak caused by an ice cream. One *S. aureus* DNA was collected from a strain isolated from ice cream and three *S. aureus* DNA from strains isolated from hand swabs of ice cream handlers. The students were instructed about methodology for DNA isolation and analysis, RAPD-PCR method, and agarose gel electrophoresis for DNA. Prior to working in the laboratory, the students were also instructed to read:

- a laboratory protocol, which consisted of introduction, utilities, and protocol of RAPD-PCR;
- a topic safety guide;
- a NTSyS-pc software guide;

The guides were carefully designed by the teachers and are included as Supporting Information. As *S. aureus* is a pathogenic bacterium, we only provided genomic DNA to students. The RAPD-PCR method used in this activity was previously carefully optimized by the teachers, taking into account reproducibility, thickness, and size of the bands. At the beginning of each laboratory session, the instructor reviewed the laboratory protocol and answered the questions asked by the students. Students who participated in this exercise should have basic biochemistry and molecular biology laboratory skills. Microbiology students are instructed on safety standards for laboratory work throughout the career. In this practical work, the teacher reviewed the safety standards to be considered to work with DNA. Students manipulated the DNA previously extracted by teacher assistants and prepared the agarose gels with the addition of SYBR Safe DNA gel stain.

Materials and Methods

Materials

Chromosomal DNAs from *S. aureus* were provided to the students. The strains were given by the teachers of Food Microbiology Research Laboratory. One *S. aureus* strain was isolated from the ice cream sample. Three *S. aureus* strains isolated from hand swabs were collected from different persons who prepare the ice cream. Strains were isolated and identified as *S. aureus* by teachers of Food Microbiology Research Laboratory. The initial manipulation of *S. aureus* strains was carried out by the teachers at the research laboratory which has the necessary safety standards for manipulate risk group two bacteria. Then, DNAs were isolated according to standard protocols [7] and Reinoso *et al.* [7]. Briefly, genomic DNA was extracted adding lysostaphin (1 mg/mL) and protease (20 mg/mL). Samples were treated with 3M NaCl–2%CTAB and extracted with chloroform–isoamyl alcohol (24:1). The DNA was precipitated with ethanol and resuspended in TE buffer (10 mM Tris–HCl [pH 8.0]; 1 mM EDTA [pH 8.0] +10 μg mL^{-1} RNAse). DNA concentration was estimated spectrophotometrically at 260 nm.

The random primer used for this assay was named P13 and its sequence is 5′-ACCGCCTGCT-3′ containing 60% of cytosine-guanine (GC) and nonpalindromic sequences (Promega), were used for DNA amplification [8].

Each group of students was also provided with the following materials and reagents: latex gloves, sterilized 1.5 mL tubes, and 0.2 mL PCR tubes (Promega, USA) microcentrifuge (Eppendorff, Germany), reagents for PCR, automatic pipettes of 2, 10, 20, and 100 μL (Gilson, USA), sterilized pipette tips,

TABLE I *Reagents for PCR master mix*

Reagent	*Final concentration*	*Volume for a mix 1× (μL)*
Buffer	10×	2.5
Cl_2Mg	3.5 mM	3.5
Primer	3 μM	3.75
Dntp	200 μM	0.2l
H_2O	–	4.85
DNA *Taq Pol*	2 U	0.2
DNA	25 ng	5

PCR rack, and ice boxes. A PCR thermocycler (MJ Research, UK) is available in the laboratory.

Laboratory equipment and reagents for PCR assay and gel electrophoresis included: automatic pipettes (10 μL), electrophoresis chamber (BioRad), power supply (BioRad), gel imaging system (MiniBis Pro, BioAmerica), 1.5% agarose gel (Promega), SYBR Safe DNA gel stain (Invitrogen), 1× buffer TBE (Tris, borate, EDTA), 100 bp molecular marker (Promega), loading dye, and distilled water.

Personal computers with NTSyS-pc software were also required for data analysis.

Methods

Class 1. PCR Reaction

At the beginning of the class, the teacher gave expository instruction about the practice, a review of PCR and an introduction to the protocol and utilization of RAPD-PCR. In addition, the teacher explained the procedure to use for extracting DNA and how to determine the appropriate concentration to use in the reaction. Each group of students carried out a RAPD-PCR assay as indicated in the protocol guide. Each group of students had a set of reagents, which they kept on ice while using it. The students were told to wear latex gloves to avoid nucleases and ribonucleases from their hands. The instructor explained the students how to do the PCR reaction mix. Then, they calculated the amount of each reagent to be added in the PCR master mix using Table I and taking into account the number of DNAs to assay. After doing the calculations, the amount of each reagent was checked with the instructor. Each group, following the order of the table, prepared the master mix in a 1.5 mL Eppendorff tube maintained on ice. After adding all the reagents, they gently mixed the reaction and span down in microcentrifuge. Finally, each group fractioned the master mix in PCR Eppendorff tubes and 5 μL of each *S. aureus* DNA template was pipetted into the tubes as follows:

Tubes 1: negative control, tubes 2: icecream sample (named IC1), tubes 3, 4, and 5: hand swab samples (named SH1, SH2 and SH3), tubes 6: positive control.

Each group prepared a negative control of the same reaction mixture with water instead of DNA template which was included in the run. In addition, students also received a DNA from a well characterized *S. aureus* strain and they used it as positive control. Risks associated with the manipulation of DNA in this activity were controlled by normal good laboratory practices.

PCR Conditions

Teacher showed the students how to adjust the thermocycler to achieve the following conditions: 95°C, 3 min; 40 × (94°C 1 min, 37°C 1 min, 72°C 1.5 min); a final extension time of 72°C, 5 min was included. Teacher assistants turned off the thermocycler and kept the tubes at 4°C until being analyzed for gel electrophoresis.

Class 2. Agarose Gel Electrophoresis

Before class, the teacher instructed students about safety for using electrophoresis equipment. Care was taken to ensure that hazards from electrical equipment, buffers and stains of DNA were understood by the students. Students were provided the molten agarose to which they added 1 μL SYBR Safe DNA gel stain per 50 mL of melted agarose. After gels were solidified, students put them into the electrophoresis chamber and poured 0.5× TBE buffer, previously diluted from 1×, into the chamber to cover the gel. Ten microlitres of each amplified products and 3 μL of DNA ladder was loaded into the wells of the agarose gel. DNA ladder was loaded into the first well. The lid was placed on the chamber and the power supply was connected at a constant voltage of 2.5 V/cm during 1 h. When the dye reached the bottom of the gel, the electricity was turned off, and the gel was observed under a gel imaging system (Mini Bis Pro, BioAmerica). Students digitalized and stored the gel images as Tagget Image Files Format (TIFF). Subsequently, the images were printed.

Class 3. Analysis of RAPD-PCR Results

In this laboratory class, we provided each student group with printouts of the electrophoresis data. Students interpreted the RAPD-PCR profiles obtained comparing amplicon sizes by visual inspection of the band profiles using the size markers on the gels. Genetic relationships were established by scoring the presence or absence of each RAPD polymorphic band. At least one computer with NT-SyS-pc software was needed, but in practice, the exercise will progress faster if there is a computer available for each student of the group. The data corresponding to the molecular weight of each band were incorporated into an Excel table. Presence was recorded with 1 and absent with 0. The percentage of similarity between two strains was estimated by using the coefficient of Dice [9]. Cluster analysis of similarity matrices was performed by unweigthed pair group

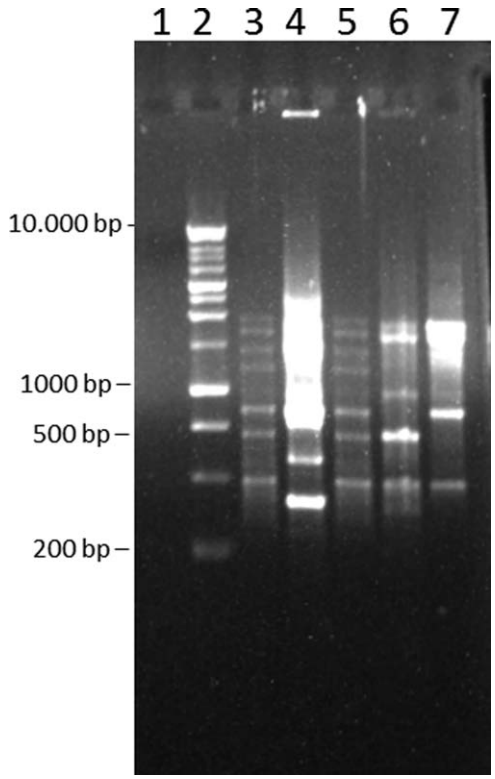

FIG 1 *RAPD-PCR profiles among S. aureus isolates of a food outbreak. Lane 1: negative control. Lane 2: 100 bp DNA ladder. Lane 3: icecream sample (IC1). Lane 4–6: hand swab samples (SH1, SH2, and SH3). Lane 7: positive control.*

method with arithmetic averages (UPGMA) [10]. When calculations were completed, a dendrogram was obtained by using the results of the RAPD assay. For these analyses, the NTSYS-pc software package (version 2.01e, Applied Biostatistics, New York, USA) was used.

The results obtained were discussed among the students and then with the teacher in order to infer the possible epidemiological links. To review what they had already learnt, the practice was evaluated with questions and a written report. The written report should be written with an introduction, materials and methods, results, and a brief discussion. It is also important to discuss the problems found during the practice and how the students would solve them.

Results and Discussion

RAPD-PCR Assay

In this laboratory practice, RAPD-PCR was used as a tool to assess the genetic relationship of four *S. aureus* strains isolated from a food outbreak in order to infer possible epidemiological links. For this practice we chose an oligonucleotide that generated an adequate numbers of bands and a limited number of low intensity bands, in order to facilitate the interpretation. RAPD profiles resulted in DNA amplification fragments ranging in size from 300 to 1500 bp. Figure 1 shows the results obtained. No amplification products were obtained in negative controls.

Analysis of RAPD-PCR Results

At the beginning of the third class, students with printouts of electrophoresis data were required to compare band patterns within a gel to complete the activity. This forced students to match band patterns and compare amplicon sizes using the size marker on each gel. By comparing the obtained bands, they attempted to identify the RAPD profiles. A set of reproducible bands produced for a particular oligonucleotide was defined as ″profile”. One group of students correctly identified all the bands, while the other group could not identify two bands. Consequently, students should be trained to be careful and alert so as to have success in the use of any molecular technique. Dendrogram constructed showed that RAPD profiles were divided into three clusters named I to III at 90% of similarity. Figure 2 shows the dendrogram constructed. Three profiles were obtained. Strains isolated from hand swabs (SH1, SH2, and SH3) belonging to clusters I–III showed to have different RAPD profiles each other. Furthermore, one isolate collected from hand swab (named SH2) shared the same RAPD profile with isolate IC1 collected from the ice cream. In conclusion, the data obtained showed that isolate SH2 collected from hand swab may be the source of the outbreak.

The postlaboratory discussion between the instructor and students, as well as the student laboratory report, showed that the students understood this technique and the relevance of this knowledge for their professional development. Furthermore, troubleshooting and limitations of the technique were discussed. In addition, we used questions to evaluate what they learnt. Examples are described below.

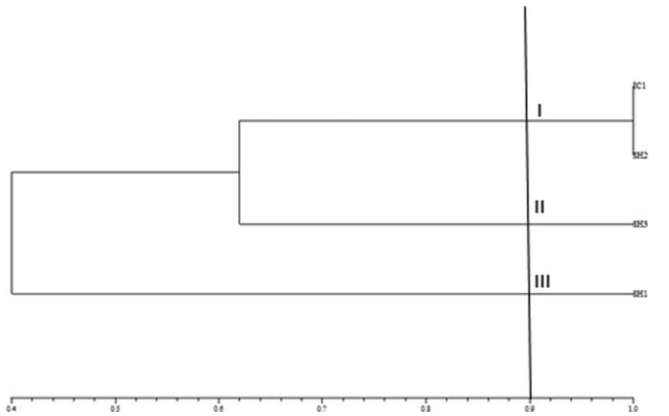

FIG 2 *Genetic relationship estimated by clustering analysis of RAPD profiles obtained with oligonucleotide OLP13. The dendrogram was generated by the unweighted pair group method with arithmetic averages (UPGMA).*

TABLE II *Students survey responses*

	Scale		
	1	*2*	*3*
Organization and management			
The course is well organized	97%	3%	–
The instructor arrives on time	98%	2%	–
Academic support			
The instructor relates course topics to each other	100%	–	–
The instructor motivates me to learn	98%	2%	–
I have been able to contact instructor when I needed	100%	–	–
Any changes in the course or teaching have been communicated effectively	100%	–	–
Overall satisfaction			
I am satisfied with the quality of the course	97%	3%	–
Laboratory exercise			
1. Do you think that you gained skills using a RAPD PCR assay?			
2. Do you consider of importance carry out RAPD-PCR assay in future Epidemiology courses?			

1: satisfactory, 2: little satisfactory, 3: unsatisfactory.

1. What is the importance of RAPD-PCR in Epidemiology? Answer: RAPD-PCR technique can be used to identify genetic fingerprints in order to infer possible epidemiologic links.
2. How does RAPD-PCR work? Answer: RAPD is a modification of the PCR, uses short oligonucleotide primers of arbitrary sequence and annealing conditions that favor nonspecific template binding.
3. What is defined as profile? Answer: A set of reproducible bands produced for a particular oligonucleotide.
4. What is the advantage of RAPD-PCR analysis over other molecular epidemiologic methods? Answer: RAPD-PCR is simple to follow, it requires no DNA sequence information for the design of specific oligonucleotides, it produces high number of fragments, it requires only small amounts of DNA and the procedure can be automated. Furthermore, unit costs per assay are low compares to other techniques.
5. How is selected an oligonucleotide for RAPD-PCR? Answer: A set of random oligonucleotides must been screening and best primers producing multiple bands are select for profiling experiments.

The answers given by the students and the written report showed that they understood the aim of this laboratory session and they could see the relevance of this technique in Epidemiology. After finishing the Epidemiologic course the student answered a survey which was designed by the Academy Commission of the Faculty. It consisted of a series of statements related to the entire course to which students responded on a 1–3 scale, where 1 is satisfactory, 2 little satisfactory, and 3 unsatisfactory. The survey covered the followings topics: Organization and management, Academic support, Overall satisfaction and Laboratory exercise. Tables II and III show the student survey responses. All the students agreed that they gained skills using RAPD-PCR technique, concluding that RAPD-PCR assay was appropriate for molecular epidemiology. Eighty seven percent of the students considered the importance of doing RAPD-PCR assay in future Epidemiology courses, while the remaining 13% suggested using another fingerprinting technique. Two students suggested performing pulse field gel electrophoresis (PFGE) instead RAPD-PCR in future Epidemiology courses. In the future, we aim to carry out a laboratory exercise doing PFGE. Although, the students felt that the laboratory module motivated them for further study and research in the field of molecular microbiology according to them they expressed during the discussion of the exercise.

TABLE III *Laboratory exercise questionnaire*

Question	*Agree*	*Not agree*
1-Do you think that you gained skills using a RAPD PCR assay?	100%	0%
2-Do you consider of importance carry out RAPD-PCR assay in future Epidemiology courses?	87%	13%*a*

[a]Two students suggested to perform pulse field gel electrophoresis instead RAPD-PCR in future Epidemiology courses.

A previous laboratory exercise has described the use of PCR methodology in order to identify unknown bacteria [11]. We attempted to use RAPD-PCR for epidemiologic molecular analysis, adopting this technique for the microbiology laboratory as a practice activity that allows students to generate and analyze data for epidemiological analysis. The RAPD PCR activity described here contributes to the training in molecular epidemiology, studying the clonal relationship between microorganisms of the same species.

Further Applications

Although this activity was designed for working with DNA from *S. aureus* strains isolated from a foodborne case, it can be reproduced by others who teach this subject using DNA from *S. aureus* obtained from different sources. Furthermore, a teacher who lacks a research laboratory in Food Microbiology can play this scheme using strains isolated from different sources or *Staphylococcus* nonpathogenic as *S. xylosus*, *S. carnosus,* or *S. equorum* from public strains collections.

Additionally, we used RAPD-PCR to assess the genetic relationship of strains isolated from different sources or hosts using a combination of primers [7].

A variety of other microbes, such as fungi, yeast, viruses, or parasites could be subjected to RAPD-PCR for genetic analysis. This technique is attractive, because of its easy of performance, its discriminative capacity and the ability to apply to any organism from a defined group or for outbreak investigation.

This work may be adaptable by the broader community because RAPD-PCR may be use to discriminate between related individuals, to study the genetic diversity, to establish genetic relationships, to contribute to plant breeding, to get DNA markers and to perform genetic analysis of populations among others. Teachers can adapt RAPD-PCR to different uses.

Conclusions

The data presented in this article, show that the RAPD-PCR provided meaningful student-generated data that can be used to draw conclusions about molecular epidemiology. The RAPD PCR activity was developed as part of an effort to introduce discipline-specific PCR experiments throughout our Microbiology career curriculum. This RAPD-PCR activity was appropriate for the microbiology laboratory, viewed as valuable and interesting by the students, and effected student learning of the application of an important molecular method. Furthermore, a variety of extensions of the basic RAPD method could also be adapted for other undergraduate courses of Microbiology Career.

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