PCR as a specific, sensitive and simple method suitable for diagnostics

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Abstract

PCR technology is a widespread method that has not reached students laboratory in anything else than a typical amplification reaction. We describe a simple application of PCR in pathogen diagnostics that enables students to identify which ampicillin-resistant organism is present in a cell culture. This experiment has been performed for one year in two “Experimental Biochemistry and Molecular Biology” courses with Biological and Chemical undergraduates. Using specific primers from the Escherichia coli β-lactamase gene, they have been able to selectively amplify a β-lactamase DNA fragment in E. coli but not in Staphylococcus aureus and, using different annealing temperatures, test the reaction specificity. By solving the “Study Questions”, students understood the specificity and sensitivity of the method, as well as the rationale that should be applied when a molecular weight pattern is used for calculating unknown DNA sizes.

1. Introduction

Practical experiments on DNA technology are currently performed in intermediate and advanced biochemistry courses, enabling the students to use the methodology and interpret results of common molecular biology techniques. In spite of the massive utilisation of PCR technique nowadays, PCR protocols have not widely reached student laboratories yet in anything else than a typical reaction, but not a real application. This prompted us to introduce Biology and Chemistry undergraduate students to some advantages of the PCR-based methods applied to clinical chemistry.

Molecular diagnostic procedures, based on the presence of a specific gene or set of genes, arise as a valuable alternative to classical methods for detection and characterisation of a pathogenic organism, therefore facilitating the prevention, control or treatment of infectious diseases. For example, the extensive use of antibiotics in the community and hospitals has fueled the appearance of bacterial resistance. Bacteria can become resistant to antimicrobial agents by a number of mechanisms [1]. The identification of the mechanism by which a pathogen organism is resistant to a given antibiotic could allow the choice of the most suitable treatment. Any useful detection strategy must be specific, sensitive and simple. Specificity means that the assay must yield a positive result just for the target organism. Sensitivity means that the assay must allow the detection of small amounts of the target organism, even in the presence of other organisms or substances. Simplicity is required for the test to be run efficiently, effectively and inexpensively on a routine basis. Pathogens were identified by procedures employing the in vitro propagation. However, cell culturing of a pathogen frequently requires a total elapsed time of days to weeks, and in some cases, pathogens will not grow. Such a method does not have the required specificity, sensitivity and simplicity — while PCR does. Moreover, PCR diminishes the risk of individual contamination inherent to any pathogen culture. A vast body of work is accumulating on the use of PCR to identify and characterise pathogenic micro-organisms [2,3]. The principle is that primers are constructed which will amplify a known sequence of a particular size from the pathogen, but will not amplify DNA from the host or from any other micro-organism. Primer sequences can distinguish between minor differences of strain and type, and as few as one molecule per million cells can be detected. Therefore, PCR amplification of a resistance gene, arises as a sensitive, specific and simple test for the detection of micro-organisms that are resistant to a given antibiotic by means of a known mechanism.

Here we describe the use of PCR to detect the presence of the β-lactamase gene in ampicillin-resistant bacteria.
and other β-lactams. β-lactamases catalyse the opening of the β-lactam ring of the antibiotic, leading to its inactivation. For this experiment, we have selected a strain of E. coli that contains the β-lactamase gene. This gene is naturally encoded in the plasmid ColE1, but for practical reasons, it is preferable to use any laboratory plasmid derived from ColE1, such as pBR, pUC, pGEM, pET or the Bluescript series. As a negative control of resistance, a bacteria sensitive to ampicillin (any E. coli laboratory strain devoid of plasmid) was employed. The use of E. coli presents several advantages in an experiment devoted to undergraduate students: (i) its wide availability in any Molecular Biology laboratory; (ii) its lack of pathological effects of the laboratory strains; and (iii) the easy-to-perform protocols available for this microorganism. To point out the selectivity of the method, comparison with a different penicillin resistant micro-organism (Staphylococcus aureus) was carried out.

From the instructor’s point of view, the experiment requires minimal preliminary preparation and is inexpensive. We have not included any prior isolation of plasmid DNA to shorten and cheapen the protocol, allowing it to be performed by students in a 7 h-session. If convenient, the practical experiment can also be divided into two shorter sessions, the first comprising PCR amplification (4 h). In a second 3 h-session, results are analysed by means of DNA agarose electrophoresis and discussed.

2. Experimental protocol

2.1. Materials, solutions, bacterial strains and culture media

The following equipment is required: automatic pipettors, disposable tips, latex gloves, UV transilluminator, Polaroid camera and film or videocam with printer, tabletop microcentrifuge, vortex, microwave oven, submarine electrophoresis facilities, Eppendorf Mastercycler 5330 or a MJ Research Minicycler, and disposable tubes (1.5 and 0.5 ml). Standard 0.5 ml tubes were preferred for PCR reactions since thin-wall 0.2 ml tubes collapsed in the students’ hands. The reagents required are: primer mix containing each oligonucleotide at 5 μM, dNTP mix containing each deoxynucleotide at 2.5 mM, 10 x PCR buffer, Taq polymerase at 5 U/μl, agarose, 10 x TBE (2 M Tris, 1 M boric acid, 50 mM EDTA), loading buffer (50% glycerol, 0.25% Bromophenol), running buffer (0.5 x TBE), 10 mg/ml ethidium bromide, molecular weight marker (λ phage digested with HindIII). The primers Amp5’ (5’-TGAAGATCAGTGGGGTGG-3’) and Amp3’ (5’-CCCCTGCTGTAGAGATA-3’) were selected with the help of the program Amplify® to amplify specifically a 690 bp band (Ac. No. X65304) from the open reading frame of the E. coli β-lactamase gene (Fig. 1A)

The bacterial strains used were E. coli DH5 as ampicillin sensitive strain, E. coli DH5 transformed with plasmid pGEM-3z as ampicillin resistant strain, and S. aureus BRL-1555 as another resistant species obtained from the Spanish Type Culture Collection. Bacteria were cultured in solid Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 2% agar) overnight a 37°C.

2.2. Selective amplifications

Amplification reactions will be carried out using 0.5 ml tubes in a volume of 25 μl containing 67 mM Tris-HCl, pH 8.8, 16.6 mM (NH₄)₂SO₄, 0.01% Tween-20, 2 mM MgCl₂, 0.1 mM each deoxynucleotide, 0.4 mM each primer, and 0.5 U of Taq polymerase (EcoGen, Spain). To perform this reaction, students have to prepare the following reaction mix for 4 tubes in a cold 1.5 ml tube: 56.4 μl of sterilised distilled water, 10 μl of 10 x PCR buffer, 4 μl of 50 mM Mg²⁺, 8 μl of dNTP mix, and 1.6 μl of 5 U/μl Taq polymerase. Four tubes are prepared with

![Figure 1](image-url)
5 µl of primer mix, and then 20 µl of the reaction mix is distributed through them. A toothpick is used to “pick” one colony of the corresponding microorganism and resuspend it in each tube as follows: E. coli sensitive to β-lactases is added in tube 1; E. coli resistant to β-lactases is resuspended in tube 2; and S. aureus resistant to β-lactases is added to tube 3. If required, a control can be carried out with the remaining reaction mix without any microorganism. Tubes are placed in the cycler programmed for 1 cycle of 1 min 92°C followed by 25 cycles of 1 min 91°C, 1 min at the annealing temperature (T_a) and 2 min at 72°C, for denaturing, annealing and primer extension phases, respectively. The last cycle was followed by 5 min at 72°C to permit the complete extension of all molecules. Two T_a are chosen: 45°C that is theoretically the minimum annealing temperature that permits specific amplification of β-lactamase gene, and 47°C that is even more stringent. Amplification products were analysed by gel electrophoresis. This was performed following a similar protocol developed in our Department [4]. Briefly, horizontal, submarine agarose gels are prepared in a simple apparatus, with 15 cm between electrodes (BioRad Mini-Sub Cell GT or Hoefer HE33). The gel tray measures 7 × 10 cm, and uses 1.5 mm-thick 8-wells combs. 40 ml of 1.0% agarose in 0.5 M NaCl 3°C, 1 min at the annealing temperature (T_a) as extension phases, respectively. The last cycle was followed by 5 min at 72°C to permit the complete extension of all molecules. Two T_a are chosen: 45°C that is theoretically the minimum annealing temperature that permits specific amplification of β-lactamase gene, and 47°C that is even more stringent. Amplification products were analysed by gel electrophoresis. This was performed following a similar protocol developed in our Department [4]. Briefly, horizontal, submarine agarose gels are prepared in a simple apparatus, with 15 cm between electrodes (BioRad Mini-Sub Cell GT or Hoefer HE33). The gel tray measures 7 × 10 cm, and uses 1.5 mm-thick 8-wells combs. 40 ml of 1.0% agarose in 0.5 × TBE are heated in a microwave oven for 2–3 min to melt down the agarose. After cooling to 50°C, 2.5 µl of ethidium bromide solution (CAUTION: highly toxic) are added. Place the running tray and comb in the gel caster, pour the agarose, and allow to solidify for 20 min. Carefully remove the gel comb and place the agarose gel in the apparatus containing 250 ml of running buffer. Samples are prepared adding 3 µl of loading buffer to each PCR reaction and then loaded. Electrophoresis is carried out for 1–2 h at constant voltage of 95 V (6 V/cm). When the dye front has migrated two-thirds of the gel, stop electrophoresis and place the gel on a UV light box and photograph (e.g. 4 s with a Polaroid 667 film at f/8 using red filter). Safety glasses, or better a full face mask, must be worn to prevent UV damage to the eyes and face.

2.3. Molecular weight calculation

Students are asked to calculate the molecular weight of the amplified bands resolved in the gel. They are guided to use the Gauss–Newton method for the least-squares fit because it is the best choice for linear regressions, once they have represented the results and rejected the outliers due to experimental error or because they are out of the range that can be fitted to a line. As far as possible, the use of Lines & Kinetics software [5] is recommended since it provides the necessary graphical interface, the handle of outliers, the minimal number of data that can estimate the same equation with a 5% error and a confidence level of 95%, the correlation of data, the standard error of correlation, and the standard error of the resulting function.

3. Results and discussion

Data presented here are class results obtained by students from the 1998–1999 course. PCR amplification was carried out using two different annealing temperatures, as described in the “Experimental protocol”. Half of the student groups used T_a = 45°C, and the other groups used T_a = 47°C. The rationale of this is to determine if the laboratory manipulations can induce the amplification of DNA fragments that are not as specific as expected. As shown in Fig. 1B, students that use the lower T_a (45°C) obtained non specific amplification in ampicillin sensitive E. coli, as well as a number of additional DNA bands in ampicillin resistant E. coli. However, no one obtained any amplification in S. aureus. The specificity of PCR amplification was greatly improved by those students that increased T_a to 47°C, obtaining an unique band of amplified DNA of about 0.65 kb size in resistant E. coli, result that is very close to the 0.69 kb expected. No band was obtained neither in sensitive E. coli, nor in ampicillin-resistant S. aureus again. From the teacher’s viewpoint, these results suggest that the practical experience should be performed in the more stringent conditions, since the time spent in waiting for the other groups to finish their reaction mixes seems to promote DNA amplifications in other locations instead of the β-lactamase gene. However, if one want to differentiate sensitive E. coli from resistant S. aureus, the T_a = 45°C must be carried out.

The students had to calculate the molecular weights of all the amplification products. The results obtained by all the groups of students were discussed in a meeting session held in the classroom. Comparison of the results obtained by groups that used different T_a pointed out the effect of an increase in this temperature in the specificity of PCR amplification. The lack of amplification in resistant S. aureus showed the species-specificity of the primers chosen. The fact that there is a common amplification band in both resistant and sensitive E. coli strains (Fig. 1B, left) has served to discuss with students the chromosomal origin of this product. Thus, both temperatures should be used to identify clearly which is the specific amplification, which are the two E. coli, and which is S. aureus.

Students were tested for their interest in this practical work, demonstrating that, in a 0–5 range, they found it very interesting (4.1), very formative (4.1) and of great novelty (4.4). The difficulty (3.3) and clarity of results (3.3) were in the same range of other practical works performed by these students. This prompted us to design new primers to evaluate other kind of species or antibiotic resistance such as kanamycin or tetracyclin.
In conclusion, we have shown that undergraduate students are able to conduct this experience in one session, enhancing the simplicity and rapidity of this technique as an alternative for classical diagnostic. Also, ampicillin resistance gene has been successfully PCR-amplified in *E. coli* by undergraduate students. It serves to help students to understand the potential application of PCR in the identification of resistant microorganisms, pointing out the specificity, sensitivity and simplicity of this technique. On the other hand, this practical experiment gets students to do calculation in the laboratory, which are reinforced by their having to answer the study questions (see below).

4. Study questions

- Calculate the molecular weight of the band obtained in the gel.
- In the case that several bands are obtained, determine which one is specific for β-lactamase, and justify the presence of common bands in several lanes.
- Infer which is the sensitive or resistant *E. coli*, and the resistant *S. aureus*.
- Explain why Amp5′ and Amp3′ do not amplify anything in *S. aureus*.

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References