Research article

Two-dimensional PCR for detecting class 1, 2 and 3 integrons

Wenwen Zhu a, Tong Wang b, Yu Zhu b, Linlin Xiao b, Wewei Liu c,**, Quhao Wei a,b,*

a School of Laboratory Medicine and Biotechnology, Southern Medical University, Guangdong Province, Guangzhou 510515, China
b Department of Laboratory Medicine, Anhui University of Science and Technology Affiliated Fengxian Hospital, 6600 Nanfeng Road, Shanghai 201499, China
c Department of Laboratory Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, 725 South Wanping Road, Shanghai 200032, China

ARTICLE INFO

Keywords:
2D-PCR
Integron
Integrase
Gene cassette
Antibiotic resistance

ABSTRACT

Integrons can capture and express foreign gene cassettes through site-specific recombination and are important genetic elements in spreading antibiotic resistance genes among bacteria. We have developed a two-dimensional PCR technology (2D-PCR) based on the base quenching probe technology in detecting three major integrons at the same time. The minimum detection limits were evaluated by detecting three plasmids each harboring different types of integron with different concentrations. The specificity of this method was evaluated by screening and typing three major types of integrons in 105 clinical Proteus isolates, and the results were compared with those of traditional PCR. Results indicated that the melting temperature (Tm) difference of the three genes was about 10 °C and was very easy to be distinguished. The minimum detection limits of intI1, intI2 and intI3 were all below 10² copies/µl. The detection results of clinical isolates were consistent with those of traditional PCR. This developed rapid, economic and high-throughput 2D-PCR based method can detect three main classes of integron at the same reaction, and can be applied to clinical isolates in large-scale integron screening and typing.

1. Introduction

Integrons are gene acquisition systems in bacterial genomes, which can effectively capture foreign genes and ensure their expression (Gil-\textit{l}ings, 2014; Kaushik et al., 2018). At the same time, integrons can be located on plasmids, transposons or chromosomes, resulting in the widespread of antibiotic resistant genes (Zhang et al., 2020). All integrons have three basic core structures, the \textit{inti} gene encoding integron integrase, the recombination site \textit{attI}, and the promoter Pc (Razavi et al., 2017). Integron integrase belongs to the tyrosine recombinase family and is also a target for screening integrons (Deng et al., 2015; Perez-Etayo et al., 2018). According to the amino acid sequence of integrase, integrons are divided into several categories, the clinically common ones are class 1, 2, and 3 integrons. Integron integrase can mediate insertion of gene cassette at \textit{attC} site and excision of gene cassette between two \textit{attC} sites (Bouvier et al., 2005). Most gene cassettes have no their own promoters and their transcription depends on the upstream common promoter Pc. According to the location, integrons can be classified into antibiotic resistant integrons (RI) on plasmids or transposons and super-integrons (SI) on chromosomes (Fluit and Schmitz, 2004). RI mainly carry gene cassettes related to antibiotics resistance, and more than 300 cassette arrays have been described (Domingues et al., 2015). As to SI, there are as many as 180 gene cassettes located within a single SI, and the functions of these gene cassettes are more complicated (Vit et al., 2021).

Polymerase chain reaction (PCR) technology is currently the most commonly used technology for screening integrons. However, traditional PCR is time-consuming and more materials are needed. Furthermore, each PCR system with a pair of specific primers can detect only one type of integron, and electrophoresis is used to identify the results (Li et al., 2018; Ren et al., 2013). 2D-PCR (Two-dimensional polymerase chain reaction technology) is a multiplex PCR detection method that combines base quenching probe technology and melting curve analysis (Zhan et al., 2018). A base quenching fluorescent probe was designed and a pre-tag sequence to identify the probe was added to the 5’ end of one specified primer. In order to identify the target genes through different melting temperature (Tm) values, some bases on the pre-tag sequence were changed. Different target genes were amplified using multiplex asymmetric PCR and identified by melting curve analysis of local double stranded DNA (dsDNA) construct of probe and single strands of PCR products. Therefore, one fluorescence channel can detect multiple target genes at the same time, and the detection throughput can be further

* Corresponding author.
** Corresponding author.
E-mail addresses: huahanvivian@126.com (W. Liu), lab_wqh@126.com (Q. Wei).

https://doi.org/10.1016/j.heliyon.2022.e11844
Received 15 April 2022; Received in revised form 29 September 2022; Accepted 17 November 2022
2405-8440/© 2022 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
increased by adding more tags and fluorescence channels. In this study, a rapid, sensitive and high-throughput 2D-PCR based method was developed to detect three main classes of integron at the same reaction.

2. Materials and method

2.1. Strains and plasmids

One hundred and five clinical Proteus isolates were collected from different specimens of patients admitted in Southern Medical University Affiliated Fengxian Hospital, Shanghai, from January 2019 to December 2019. Class 1 integron integrase gene (intI1) positive plasmid pHSint1 was collected (Wei et al., 2011), and class 2 integron integrase gene (intI2) positive plasmid pINTI2P was constructed in our laboratory (Wang et al., 2021). According to the sequence of class 3 integron integrase gene (intI3) in the GenBank with accession No. LC31665, primers int3-1, int3-2, int3-3 (Table 1) were designed. Then, the three primers were connected by overlapping PCR. The PCR products were connected to pMD19 plasmid by using T-A cloning kit (TaKaRa, Japan), and verified by sequencing, the constructed plasmid was named pMDintI3. Escherichia coli DH5α was used as negative control. Proteus mirabilis 47437 (containing intI1 and intI2) was maintained in our laboratory (Wei et al., 2014).

2.2. DNA extraction

Isolates were grown in Lysogeny Broth (LB) at 37 °C with shaking for 14 h, to inhibit the growth of miscellaneous bacteria, when necessary, with ampicillin (100 μg·ml⁻¹). Genomic DNA from Proteus isolates was extracted using the SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech, China) according to its protocol. Plasmid DNA from E. coli DH5α was extracted using the Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, China). The extracted DNA was stored at −20 °C for subsequent analysis.

2.3. 2D-PCR

2D-PCR was performed with a LightCycler 480II (Roche, Germany). Each PCR was performed in a 25 μl reaction mixture containing 2.5 μl 10 × PCR buffer (Mg²⁺ free), 1.5 μl Mg²⁺ (25 mM), 0.7 μl dNTP Mixture (2.5 mM each), 0.5 μl Ex Taq HS (5 U·μl⁻¹) (TaKaRa, Japan), 0.1 μl each of pre-tag harboring primers intI1-LC1, intI2-LC2 and intI3-LC1 (10 μM), 0.6 μl each of primers intI1-LC5, intI2-LC3 and intI3-LC2 (10 μM), 0.4 μl intI-P (10 μM), 15.3 μl deionized water (HPLC grade) and 2 μl extracted genomic DNA (about 100 ng·μl⁻¹) or plasmids with different copy numbers. A blank control (distilled water), a negative control (E. coli DH5α) and positive controls (P. mirabilis 47437 and E. coli DH5α containing pMDintI3) were included in each run. The reaction was performed under the following conditions: denaturation for 4 min at 95 °C; 40 cycles of 10 s at 95 °C, 30 s at 60 °C; followed by melting curve analysis with temperature range from 30 °C to 80 °C, with a transition rate of 0.1 °C s⁻¹, and continuous detection of the fluorescence of the dsDNA constructed of probe and single strands of PCR products. All 2D-PCR reactions were performed in triplicate.

2.4. Traditional PCR in integron screening

Class 1, 2 and 3 integrons were screened by traditional PCR in 105 Proteus isolates, where extracted genomic DNA served as template. using primers intF and P2R2, int2F and int2R, int3F and int3R for amplifying intI1, intI2 and intI3 respectively. Each PCR was performed in a 20 μl reaction mixture containing deionized water (HPLC grade) 8.2 μl Premix Taq (TaKaRa, Japan) 10 μl, primers (10 μM) 0.4 μl each, and genomic DNA (about 100 ng/μl) 1 μl. PCR was used with the following cycling conditions: 94 °C for 4 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 40 s, and finally 72 °C for 5 min on a Veriti thermal cycler (Thermo, USA), the PCR products were analyzed by electrophoresis on a 0.8% agarose gel. Positive controls (P. mirabilis 47437 for intI1 and intI2, E. coli DH5α containing pMDintI3 for intI3), negative control (E. coli DH5α) and blank control (deionized water) were included in each run.

3. Results

3.1. Specificity

To clarify the specificity of this 2D-PCR based method, samples containing single intI gene (pHSint1, pINTI2P or pMDintI3), two intI genes (pHSint1 and pINTI2P, pHSint1 and pMDintI3, or pINTI2P and pMDintI3), or a mixture of three intI genes (pHSint1, pINTI2P and pMDintI3), with concentrations about 10⁷ copies·μl⁻¹ for each plasmid, were subjected to this method. As shown in Figure 1, the Tm values of

Table 1. Primers and probes used in this study.

| Primer | Sequences (5'→3') | location | Reference |
|--------|-------------------|----------|-----------|
| intF   | CCAGGCGTCTGCGGTAAATAC | intI | This study |
| P2R2   | CGGACGGCATAGACGTA | intI | This study |
| int2R  | CGGGATATGCGACAAAAAGGT | intI2 | This study |
| int2F  | GTACGAAACGAGTGGAAATG | intI2 | This study |
| int3R  | TGGCGAACAAGCTGATCTGTC | intI3 | This study |
| int3F  | TGGAGGGCTTCTCCGGAGGAC | intI3 | This study |
| int3-1 | GGAGGCTGTCGGCTTGTCCTGGCAGCAAGTGII | intI3 | This study |
| int3-2 | GTGGCGAAGGCTTGGACAGATACGTTGTTGGCAATGCGACGGCTGAACCTGACCTT | intI3 | This study |
| int3-3 | GCACCTGGTTGGAGGAAAGCAGTGAGCGGCAACTAAAAAGGGTTAGTTCAGGGCT | intI3 | This study |

Δ The pre-tags of primers were underlined, point mutations in the tags were marked in bold letters.
target \textit{intI1} (Figure 1A), \textit{intI2} (Figure 1B) and \textit{intI3} (Figure 1C) genes were approximately 46 °C, 66 °C and 56 °C respective, and the results were easy to interpret. The same results were obtained in samples containing two (Figure 1D, 1E, 1F) or three (Figure 1G) different \textit{intI} genes and there was no cross reaction between different types.

3.2. Minimum detection limit

In the minimum detection limit experiment, plasmid samples with series concentrations of $10^7$–$10^2$ copies·μl$^{-1}$ were conducted in this method. As shown in Figure 2, when class 1 (Figure 2A), 2 (Figure 2B), 3.2. Minimum detection limit

In the minimum detection limit experiment, plasmid samples with series concentrations of $10^7$–$10^2$ copies·μl$^{-1}$ were conducted in this method. As shown in Figure 2, when class 1 (Figure 2A), 2 (Figure 2B),
and 3 (Figure 2C) integrons plasmid samples were $10^2$ copies/$\mu$l, 2D-PCR could distinguish the three integrons well.

3.3. Applied in clinical samples

In order to evaluate the application of this 2D-PCR based method, clinical Proteus isolates were included in this study. In traditional PCR results, of the 105 Proteae collected, 53 harbored a class 1 integron and 83 a class 2 integron. No class 3 integrons were detected at the same time, there were 53 strains positive for both class 1 and class 2. Results of 2D-PCR showed that in 105 clinical Proteus isolates, intI1 was positive in 53 isolates, intI2 was positive in 83 isolates, while intI3 was negative in all 105 isolates (Figure 3). Of them, both intI1 and intI2 were positive in 45 isolates. The results of 2D-PCR were consistent with traditional PCR.

4. Discussion

The discovery of antibiotics provides a powerful weapon for clinical anti-infection treatment, but the wide use of antibiotics leads to antibiotic resistance and produces a large number of multiantibiotic resistant strains (de Vries et al., 2019). Antibiotic resistance may accelerate mankind get into the post antibiotic era (Mc Carlie et al., 2020). Integron is a bacterial genetic element that can capture foreign antibiotic resistance gene cassettes through its own platform, resulting in the spread of antibiotic resistance (Zhang et al., 2020), therefore it plays an important role in the spread of antibiotic resistance genes, especially in Gram-negative bacteria (Escudero et al., 2015). The specific process of integron excision and integration of resistance gene cassettes in vitro has not been successfully established, but this process was unmasked to some extent. For example, the process of integron mediated gene cassettes excision and integration are directly related to SOS response, and repressor LexA controls the expression of integrase and regulates the recombination of gene cassettes (Guérin et al., 2009). The integron integrase preferentially binds to the bottom chain of the single-stranded attC, and the extra-helical Bases (EHBs), Unpaired Central Spacer (UCS) and Variable Terminal Structure (VTS) affects chain selection (Francia et al., 1999; Nivina et al., 2016). Furthermore, attC hairpins have a conserved high GC-content, which can create a dynamic equilibrium between attC fully opened by SSB and a partially structured attC-6-SSB complex, which is recognized by the integrase IntI (Grieb et al., 2017). With the understanding of integrons, some integron mediated properties become very important for human health, such as increased virulence, pathogenicity or antibiotic resistance of new antibiotics. At the same time, integron is a platform for gene assembly and thus, it has broad application prospects in the fields of industry, medicine, biotechnology and synthetic biology (Ghaly et al., 2020).

Integrons have both advantages and disadvantages for human beings, therefore it is of great significance to find and study them. At present, the method of screening integrons involves screening integrase and then the gene cassettes inserted into integrons are analyzed. PCR and PCR-related methods are mainly used under such circumstances (Li et al., 2018). In the past few decades, multiplex PCR and real-time PCR have been widely used for the identification of integrons. Multiplex PCR method have been used to detect integrons in Staphylococcus aureus, however, this method requires agarose gel electrophoresis to identify the amplified PCR products, which is time-consuming (Ren et al., 2013). In this method, 2D-PCR assays were depended on the melting temperatures (Tm) of the amplicons to differentiate genes. In order to distinguish the three integrons easily, several point mutations were made in the tags of pre-tag harboring primers (Table 1). It can be seen that the melting temperature difference of the three genes was about 10 °C, which was very easy to distinguish (Figure 1). The minimum detection limits of intI1, intI2 and intI3 were below $10^5$ copies/$\mu$l respective, and 2D-PCR can

Under the condition of rational design of probe, tag and primer sequence, 2D-PCR can detect 30 target genes simultaneously (Zhan et al., 2020). In this method, 2D-PCR assays were depended on the melting temperatures (Tm) of the amplicons to differentiate genes. In order to distinguish the three integrons easily, several point mutations were made in the tags of pre-tag harboring primers (Table 1). It can be seen that the melting temperature difference of the three genes was about 10 °C, which was very easy to distinguish (Figure 1). The minimum detection limits of intI1, intI2 and intI3 were below $10^5$ copies/$\mu$l respective, and 2D-PCR can
distinguish the three integrons well at 10^2 copies/μl (Figure 2A, 2B, 2C). The detection results of clinical isolates were consistent with traditional PCR. All these results showed that 2D-PCR constructed in this study had high specificity and sensitivity. It can classify three integron types at the same time, and can be applied to clinical integron screening.

In summary, we have established a rapid, economic and high-throughput 2D-PCR based method to detect three main classes of integron at the same reaction. This method can be applied to clinical isolates in large-scale integron screening and typing.

Declarations

Author contribution statement

Wenwen Zhu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Tong Wang, Yu Zhu: Performed the experiments; Analyzed and interpreted the data.
Linlin Xiao: Contributed reagents, materials, analysis tools or data.
Weimei Liu: Conceived and designed the experiments.
Quhao Wei: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

Mr Quhao Wei was supported by Construction of Key Medical Specialties in Shanghai [ZK2019B29], the National Natural Science Foundation of China [No. 81572034].

Mrs Weimei Liu was supported by Special Clinical Research Project of Shanghai Municipal Health Commission [202140147], Outstanding Academic Leaders Plan of Shanghai [2018BR07].

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Barraud, O., Baclet, M.C., Denis, F., Ploy, M.C., 2010. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J. Antimicrob. Chemother. 65 (8), 1642–1645.

Bouvier, M., Demarre, G., Mazel, D., 2005. Integron cassette insertion: a recombination process involving a folded single strand substrate. EMBO J. 24 (24), 4356–4367.

de Vries, M., Kenis, P., Kraaij Dokkruyweg, M., Ruitenberg, E.J., Raab, J., Timen, A., 2019. Collaborative emergency preparedness and response to cross-institutional outbreaks of multidrug-resistant organisms: a scenario-based approach in two regions of The Netherlands. BMC Publ. Health 19 (1), 52.

Deng, Y., Bao, X., Ji, L., Chen, L., Liu, J., Xiao, J., Chen, D., Bian, H., Li, Y., Yu, G., 2015. Resistance integrons: class 1, 2 and 3 integrons. Ann. Clin. Microbiol. Antimicrob. 14, 45.

Domingues, S., da Silva, G.J., Nielsen, K.M., 2015. Global dissemination patterns of common gene cassette arrays in class 1 integrons. Microbiologyology 161 (7), 1313–1337.

Escudero, J.A., Lout, C., Nivina, A., Mazel, D., 2015. The integron: adaptation on demand. Microbiol. Spectr. 3 (2). MDNA3-0019-2014.

Fluit, A.C., Schmitz, F.J., 2004. Resistance integrons and super-integrons. Clin. Microbiol. Infect. 10 (4), 272–288.

Francis, M.V., Zabala, J.C., de la Cruz, F., Garcia Lobo, J.M., 1999. The IntI1 integrase preferentially binds single-stranded DNA of the attC site. J. Bacteriol. 181 (21), 6844–6849.

Ghaly, T.M., Geoghegan, J.L., Tett, S.G., Gilings, M.R., 2020. The Peril and Promise of Resistance integrons: beyond antibiotic resistance. Trends Microbiol. 28 (6), 455–464.

Gilings, M.R., 2014. Integrons: past, present, and future. Microbiol. Mol. Biol. Rev. 78 (2), 257–277.

Grieß, M.S., Nivina, A., Cheeseman, B.L., Hartmann, A., Mazel, D., Schlierf, M., 2017. Dynamic stepwise opening of integron attC DNA hairpins by SSB prevents toxicity and ensures functionality. Nucleic Acids Res. 45 (18), 10555–10563.

Guerin, E.C.G., Sanchez-Alberola, N., Campoy, S., Erill, I., Díaz Re, S., Gonzalez-Zorn, B., Barbe, J., Ploy, M.C., Mazel, D., 2009. The SOS response controls integron recombination. Science 324 (5930), 1034.

Hardwick, S.A., Stokes, H.W., Findlay, S., Taylor, M., Gilings, M.R., 2008. Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. FEMS Microbiol. Lett. 278 (2), 207–212.

Kausik, M., Kumar, S., Kapoor, R.K., Virdi, J.S., Gulati, P., 2018. Integrons in Enterobacteriaceae: diversity, distribution and epidemiology. Int. J. Antimicrob. Agents 51 (2), 107–176.

Li, Y., Yang, L., Fu, J., Chen, D., Zhang, L., 2018. Genotyping and high flux sequencing of the bacterial pathogenic elements - integrons. Microb. Pathog. 116, 22–25.

Mc Carlie, S., Boucher, C.E., Bragg, R.R., 2020. Molecular basis of bacterial disinfectant resistance. Drug Resist Update 48, 100672.

Nivina, A., Escudero, J.A., Viteri, C., Mazel, D., Lout, C., 2016. Efficiency of integron cassette insertion in correct orientation is ensured by the interplay of the three unpaired features of attC recombination sites. Nucleic Acids Res. 44 (16), 7792–7803.

Perco Enayo, L., Berzosa, M., Gonzalez, D., Vitas, A.L., 2018. Prevalence of integrons and insertion sequences in ESBL-Producing E. coli isolated from different sources in Navarra, Spain. Int. J. Environ. Res. Publ. Health 15 (10), 2308.

Razavi, M., Marathe, N.P., Gilings, M.R., Flach, C.F., Kristiansson, E., Joakim Larsson, D.G., 2017. Discovery of the fourth mobile sulfonamide resistance gene. Microbiomes 5 (1), 160.

Ren, C., Zhao, Y., Shen, Y., 2013. Analysis of the effect of integrons on drug-resistant Staphylococcus aureus by multiplex PCR detection. Mol. Med. Rep. 7 (3), 719–724.

Viti, C., Richard, E., Fournes, F., Whiteway, C., Eyer, X., Lapailleire, D., Parisi, V., Mazel, D., Lout, C., 2021. Cassette recruitment in clinical Proteus mirabilis isolates. J. Antimicrob. Chemother. 76 (8), 212–225.

Vit, C., Richard, E., Fournes, F., Whiteway, C., Eyer, X., Lapaillerie, D., Parisi, V., Mazel, D., Lout, C., 2021. Cassette recruitment in the chromosomal Integron of Vibrio cholerae. Nucleic Acids Res. 49 (10), 5654–5670.

Wang, M., Cheng, A., 2020. Dissemination of antibiotic resistance genes (ARGs) via integron harboured gene cassette impacts integration efficiency in class 1 integron. Mol. Microbiol. 80 (3), 1326–1336.

Zhan, Y., Zhang, J., Yao, S., Luo, G., 2020. High-throughput two-dimensional polymerase chain reaction technology. Anal. Chem. 92 (1), 674–682.

Zhang, S., Abbas, M., Rehman, M.U., Huang, Y., Zhou, R., Gong, S., Yang, H., Chen, S., Wang, M., Cheng, A., 2020. Dissemination of antibiotic resistance genes (ARGs) via integrons in Escherichia coli: a risk to human health. Environ. Pollut. 266 (Pt 2), 115260.