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Case Report

Development of a candidate method for forensic microbial genotyping using multiplex pyrosequencing combined with a universal biotinylated primer

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ABSTRACT

Bacterial genotyping can be used for crime scene investigations and contribute to the attribution of biological attacks for microbial forensics. PyroMark ID Pyrosequencer as an accurate detection platform for single nucleotide polymorphisms (SNPs) has been applied to identify and resolve microorganisms involved in closely *Escherichia coli* O157:H7 (*E. coli* O157:H7). To explore more applications and improve the efficiency for pyrosequencing in this field, we developed a method integrated multiplex pyrosequencing with a universal primer. Two multiplex pyrosequencing assays with a universal biotinylated primer were designed to analyze five SNPs located in four gene of *E. coli* O157:H7 strain. The accuracy of the established assays was validated by genotyping reference strain *E. coli* O157:H7 EDL933 and *E. coli* K-12. We also demonstrated that two multiplex pyrosequencing assays were specific and sensitive for genotyping closely related *E. coli* O157 strains. Reproducibility of results and multiplexing capability were evaluated by a comparison of this method with the monoplex pyrosequencing. Furthermore, these two multiplex pyrosequencing assays have been successfully applied to detect 11 *E. coli* O157 strains isolated from 1504 Chinese livestock samples. This method reduces costs and time consumption in the process of pyrosequencing analysis, and potentially serves as a rapid tool and reliable candidate strategy for the microbial identification and other genotyping application.

1. Introduction

Microbial forensics, posing the next great forensic challenge, is a rapidly evolving scientific discipline from the anthrax mailings [1,2]. In this new area, many microbes including *Bacillus anthracis*, *Yersinia pestis*, especially a number of foodborne pathogens like *Escherichia coli* O157:H7 (*E. coli* O157:H7), might potentially be used as bioterrorist agents in an attack [3]. During the bioterrorism criminal investigation of aforementioned microbes, the ultimate goal of attribution for microbial forensics is to discriminate individual isolates of a particular microbe so as to uniquely identify the source. Hence, there is a need for establishing forensic investigative methods to identify the precise strain and substrain, rather than just to the species level, which might meet the requirement for epidemiological investigation. A good example using of forensic strain subtyping analysis is the determination that the *B. anthracis* from the 2001 anthrax-letter biocrime in the United States were the Ames strain toward laboratory sources, not just the identification of the pathogen in the species level for epidemiological investigation. However, some existing traditional analyses including 16S rDNA sequencing may effectively discriminate bacteria at the species level, but they lack the resolving power to differentiate individual strains. For example, the genetic information from 16S rDNA was unable to differentiate strains within the same species, such as closely related *E. coli* O157:H7 strains. So microbial forensics will employ to establish molecular techniques and newer advanced methods, which still may be under development for identifying a bioattack organism in greatest

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detail. According to the requirement of reliable and valid microbial forensic methods, some progress has been made in molecular genetic typing and analysis strategy focused on characterizing microbial forensic evidence, which play an important role in investigating potential biocrimes [4].

During the last three decades, *E. coli* O157:H7 as a common food-borne pathogen caused severe illness with bloody diarrhea and haemolytic uremic syndrome throughout the world. In China, the extraordinarily large outbreaks of *E. coli* O157:H7 occurred in Xuzhou and resulted in 195 haemolytic uremic syndrome and 177 death in 1990. Especially, frequent outbreaks of *E. coli* O157:H7 strains demonstrated clear difference in the virulence and variation in disease severity although almost all of them are virulent. As it is impossible to anticipate the next organism used for biocrime, we need not only to concentrate on the most common potential microbe such as *E. coli* O157:H7, but also to pay more attention to different virulent *E. coli* O157:H7 substrains for precise forensic attribution. Moreover, bioengineering could also be appealing to some bioterrorists from microbial forensic perspective, just as a nonpathogenic *E. coli* transformed by bioengineering may produce a widespread outbreak like *E. coli* O157:H7 [2]. Thus molecular typing methods are increasingly focused and used to assess the relatedness of this "bioterrorist" strain or quickly screen possible bioengineering strains for the potential of an attack. Recent research in the genetics of *E. coli* O157:H7 has revealed that single nucleotide polymorphisms (SNPs) can resolve closely related bacterial genotypes and act as useful subtyping biomarkers for microbial forensic investigations [5,6]. Particularly canonical SNPs analyzed by many new DNA sequence-based methods could rapidly establish accurate phylogenetic positioning [7,8]. Among these aforementioned molecular subtyping techniques, pyrosequencing has become a useful tool for mutation analysis, copy-number variation studies, DNA methylation analysis and microbial identification [9–12]. Multiple detection platforms based on pyrosequencing technique were developed and widely applied, such as interrogating genomic diversity of *E. coli* O157:H7 and determining SNP allele frequency by PyroMark ID Pyrosequencer [13]. It has been proved that PyroMark ID Pyrosequencer as medium-throughput SNP genotyping platform is much more accurate than Sanger sequencing in identifying SNPs [14].

Pyrosequencing not only has the potential advantage of accuracy, ease-of-use and high flexibility, but also show the methodological characteristics of easy to extend. Most importantly, multiplex pyrosequencing could perform in the presence of single or multiple templates with several sequencing primers [15]. Some recent studies demonstrated the use of this multiplex pyrosequencing in microbial SNPs genotyping, for example four *B. anthracis* specific rpoB SNPs were detected in duplex sequencing reactions [16,17]. Microbial community analysis can easily be completed through pyrosequencing of 16S rRNA gene multiplex amplicons on a single run [18]. Multiplex pyrosequencing that can effectively improve throughput has been confirmed as a powerful microbial forensic tool. Another methodological development of pyrosequencing is the application of universal primers to purify PCR-generated single-strand DNA with biotin as a sequencing template [19]. By using one universal primer, alternative chemically tagged labeled DNA fragments can be generated without the sequence-specific biotinylated primers for each DNA variant. So universal primer overcomes cost and time consumption from synthesis of a biotinylated sequence-specific primer, which limits the application of pyrosequecing [20,21].

As mentioned previously, it is necessary for *E. coli* O157:H7 to develop reliable and valid methods to detect genomic diversity. Considering the recent approach for pyrosequencing analysis, the aim of this study was to explore more efficient solution for identifying individual strains of *E. coli* O157:H7 based on PyroMark ID Pyrosequencer. We developed a method for forensic microbial genotyping using multiplex pyrosequencing combined with universal primers, and then we validated this method by examining SNPs in *E. coli* O157 strains from China.

2. Materials and methods

2.1. DNA samples and quantification

DNA extracted from *E. coli* O157:H7 reference strain EDL 933 acted as positive control sample was kindly provided by a Chinese expert in microbiological research. At the same time, DNA from other 11 *E. coli* O157 strains (consist of 7 *E. coli* O157:H7 and 4 *E. coli* O157:H?) isolated from 1504 Chinese livestock samples as described in detail elsewhere [22] was also supplied. DNA was isolated from *E. coli* K-12 strain which was purchased from China General Microbiological Culture Collection Center and cultured to act as reference sample of different type. Chromosomal DNA was quantified using NanoDrop ND-1000 UV spectrophotometer (Thermo scientific, Wilmington, DE, USA) and diluted to a concentration of 10 ng/μl.

2.2. Primer of PCR and pyrosequencing design

The reference sequence (NC_002695) of *E. coli* O157:H7 strain Sakai was used for our primer design. The primer pairs amplified ECs0654, ECs2357, ECs2521 and ECs3881 genes [7] in whole genome sequence of *E. coli* O157:H7 strain Sakai and pyrosequencing primers were designed with the SNP Primer Design Software (Biotage AB, Uppsala, Sweden). It was described that the process of designing and performing two multiplex pyrosequencing assays for analyzing differential SNP in the previously four genes of *E. coli* O157:H7. The multiplex 1 interrogates two SNP sites named ECs0654-125 and ECs0654-281 residing on the same amplicon. The other assay, multiplex 2, simultaneously examines the ECs2357-539, ECs2521-1060 and ECs3881-438 residing on separated amplicons. Firstly, entering the DNA sequence into the PSQ Assay Design 1.0.6 software (Biotage AB, Uppsala, Sweden), we chose the sequence-specific PCR primers and pyrosequencing primer with high scores and different 3′-ends according to the suggestion from the software. Secondly, entering the sequence to analyze into the PyroMark ID 1.0 software (Biotage AB, Uppsala, Sweden), the optimal dispensations and theoretical peak patterns were obtained. Peaks were shown as black and gray bars to differentiate signal from different primer extensions.

2.3. Defining the biotinylated universal primer

The biotinylated universal primer was 5′ biotin-ATCTGTGCCGAGGCTCAGGC and purified by HPLC [19]. The tailed sequence of the universal primer (GTGCCGAGGCTCAGGC) was complementary to the universal primer and added on the 5′ terminus of any one of the aforementioned sequence-specific PCR primer. The previous sequence of the universal primer was also added on the 3′ terminus of the template strand, and then reanalyzed the sequence with the SNP Primer Design Software to ensure no 3′ end loop formations from the addition of the universal primer sequence. During the process of example for a single PCR amplification, three kinds of primers were a biotinylated universal primer, a tailed sequence-specific primer and another sequence-specific primer. Two sequence-specific primer initiated PCR amplification of the genomic DNA, and these initial amplified fragments served as templates for the biotinylated universal primer and the tailed sequence-specific primer in subsequent PCR amplifications to produce labeled amplicons. The sequences of the whole primers were shown in Table 1.