Molecular and Cellular Probes 24 (2010) 396–400

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Rapid and sensitive detection of *Enterobacter sakazakii* by cross-priming amplification combined with immuno-blotting analysis

Zhao Yulong [a,1], Zhang Xia [b,1], Zhang Hongwei [b], Liu Wei [b], Zheng Wenjie [b,**], Huang Xitai [a,*]

[a] College of Life Sciences, Nankai University, Tianjin 300071, China
[b] Tianjin Entry-Exit Inspection and Quarantine Bureau, Tianjin 300457, China

ARTICLE INFO

Article history:
Received 29 August 2010
Accepted 8 September 2010
Available online 17 September 2010

Keywords:
Enterobacter sakazakii
Cross-primer amplification (CPA)
Immuno-blotting analysis

ABSTRACT

Enterobacter sakazakii is a widespread and life-threatening bacterium especially in polluted powdered infant milk formula. Several methods have been developed for detection of *E. sakazakii* such as physiological and biochemical methods, PCR and loop-mediated isothermal amplification. However, these procedures were disadvantages due to a long assay time, low sensitivity or the use of toxic reagents. Our method of cross-priming amplification (CPA) under isothermal conditions combined with immuno-blotting analysis made the whole detection procedure more sensitivity and lower time-consuming. A set of specific displacement primers, cross primers and testing primers were designed based on six specific sequences in *E. sakazakii* 16S-23S rDNA internal transcribed spacer. Under isothermal condition at 63 °C for 60 min, the specific amplification and hybridization steps were processed simultaneously. The specificity of the CPA was tested in panel of 54 different bacterial strains and 236 milk powder products. Two red signal lines were developed on the BioHelix Express strip in all of positive *E. sakazakii* strains, and only one signal line was demonstrated by non- *E. sakazakii* bacterial strains. The limit of decetion of CPA was 6.3 ± 2.7277 fg for the genomic DNA, 88 ± 8.7892 cfu/ml for pure bacterial culture, and 3.2 ± 2.0569 cfu per 100 g milk powder with pre-enrichment. The current study demonstrated that the assay method of CPA combined with immuno-blotting analysis was a specific and sensitive detection for the rapid detection of *E. sakazakii*.

1. Introduction

Enterobacter sakazakii, which was known as "yellow pigmented *Enterobacter cloacae*", was classified as a unique species by Farmer in 1980, based on molecular differences and biochemical detection [1]. *E. sakazakii* is an opportunistic pathogen that can cause life-threatening disease of neonatal meningitis, bacteraemia, necrotizing enterocolitis (NEC) and sepsis [2,3], with mortality rates of 40–80% [4,5]. It reported that the rates of low-birth weight or immuno-compromised infants would be increased after infected by this bacterium [6]. Additionally, it was found that *E. sakazakii* could be isolated from almost all the environment and from a diverse range of foods [7–11], especially powdered infant milk formula (PIF) [12,13], which indicated that *E. sakazakii* is a widespread bacteria.

Several methods have been reported for the identification of *E. sakazakii*. The traditional physiological and biochemical procedures recommended by the U. S. Food and Drug Administration (USFDA) are laborious and poor efficiency (at least 7 days for final results). With the introduction of the molecular biology, a polymerase chain reaction (PCR) detection method has been developed [14]. But this protocol was restricted by the laboratory apparatus and also time-consuming by taking at least 3 h. A loop-mediated isothermal amplification (LAMP) method has been developed by Liu et al. [15], which detected *E. sakazakii* without asking for the specific equipment and could finish the examination in 2 h. However, the weak point of this method was the analysis and confirmation step. The products of LAMP were always analyzed by agarose gel electrophoresis and restricted endonuclease digestion. It would take a long time and have to use toxic reagent (ethidium bromide) stain the gel.

Cross-priming amplification (CPA) is a novel technique and its mechanism was described by Fang et al. [16]. Using six to eight primers, the sensitivity and specificity of the detection procedure is increased under isothermal conditions within 1 h. Besides the advantage of sensitivity and efficiency, the highlight of the method

* Corresponding author. Tel./fax: +86 22 23508874.
** Corresponding author. Tel./fax: +86 22 66273178.
E-mail addresses: zhengwj@tjciq.gov.cn (Z. Wenjie), huangxt@nankai.edu.cn (H. Xitai).
[1] Authors made the same contribution.

0890-8508/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2010.09.001

is the simple analytic procedure. It requires only a water bath without toxic reagent and just takes five to ten minutes to get the final results with an immuno-blotting assay by BioHelix Express strip (BESt). In CPA assay, two testing primers are involved in the isothermal amplification and labeled with biotin and FITC, respectively. As a positive test, the end of the amplicon labeled with biotin binds the colloidal gold, and the other end labeled with FITC hybridizes anti-FITC antibody which had been located on the test line of the strip, resulting in the red colloidal gold accumulated at the test line in 5–10 min. The purpose of this study was to develop a new cross-priming amplification method for *E. sakazakii* in the food sample, based on 16S–23S rDNA internal transcribed spacer (ITS).

2. Materials and methods

2.1. Bacterial strains and genomic DNA extraction

In this study, 22 *E. sakazakii* strains, with 4 ATCC and 18 strains isolated from food and human, and 32 other bacteria were tested (Table 1). All of the food and clinical samples were identified by Tianjin Entry-Exit Inspection and Quarantine Bureau, P. R. China (CIQ Tianjin), according to the USFDA standard methods. Bacterial genomic DNA was extracted from each strain by Wizard Genomic DNA Purification Kit (Promega, Inc., Madison, USA), and stored at −20 °C for use.

2.2. PCR reaction conditions

PCR reaction was performed in a total 25 μl reaction system containing 12.5 μl of 2×PCR Master Mix (0.05 units/μl *Taq* DNA polymerase, 4 mM $MgCl_2$, 0.4 mM of each dNTP) (Fermentas), 0.5 μM each primer (forward primer 5′- GGGTTGTCTGCGAAAGC-GAA -3′ and reverse primer 5′- GTCTTCGTGCTGCGAGTTTG -3′) and 1 μl of certain concentration DNA template. After a 5-min denaturation at 95 °C, the PCR mixtures were subjected to 35 cycles of amplification at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and final amplification at 72 °C for 10 min. The PCR products were electrophoresis on 1.5% agarose gel.

2.3. Primers for cross-priming amplification (CPA)

The primers for CPA were listed on Table 2.

2.4. CPA reaction conditions

CPA reaction was carried out in a total 20-μl reaction mixture containing 0.4 μM each of CF and CR, 0.8 μM each of DFs and DRa, 0.1 μM each of DP1s and DP2a, 0.5 mM dNTPs (Fermentas), 2 μl 10×Bst buffer, 6 units of Bst DNA polymerase large fragment (New England Biolabs), 4 mM $MgSO_4$ (Sigma), 0.5 M betaine (Sigma), and 1 μl appropriate amount of target DNA. The CPA reaction was carried out at 63 °C for 1 h and stored at 4 °C.

2.5. Analysis of CPA products

When CPA amplification finished, 6 μl product of each reaction was dipped on the BESt strip (Ustar Biotech Co., Ltd., Hangzhou, China), then the strip was taken into the assay buffer. After the CPA product was hybridized with anti-biotin and anti-FITC antibody for 10 min, the positive result demonstrated two red lines at the test and control line position, respectively. And the strip of negative reaction had only control line. The rest product was cloned and sequenced by Sangon, China.

Table 1
Bacterial strains for CPA detection.

Species	Strain	Origin
Enterobacter sakazakii	ATCC 12868	Unknown
	ATCC 29004	Unknown
	ATCC 29544	Unknown
	ATCC 51329	Unknown
	ENS 5329	Milk powder, Ukraine
	ENS 5413	Milk powder, America
	ENS 5729	Fish meal, Japan
	ENS 51024	Milk powder, India
	ENS 51107	Whey powder, Holand
	ENS 51227	Milk powder, New Zealand
	ENS 51229	Milk powder, Australia
	ENS 6607	Milk powder, Ireland
	ENS 70115	Whey powder, France
	ENS 70216	Infant milk powder, China
	ENS 70307	Whey powder, Poland
	ENS 71123	Milk powder, Canada
	Saka 100323	Whey protein powder, Argentina
	Saka 081013	Milk powder, Australia
	Saka 090318	Butter cheese, Australia
	Saka 090224	Chocolate cake mix, America
	RFS	Fish meal, Pakistan
	SHY	Puffing food, China
Enterobacter aerogenes	ATCC 13048	Unknown
	CGMCC 1.876	Unknown
	CGMCC 1.489	Unknown
Enterobacter cloacae	ATCC 13047	Unknown
	CGMCC 1.81	Unknown
	CGMCC 1.1733	Unknown
	CMCC 45301	Unknown
Enterobacter intermedium	E.int 51231	Milk powder, China
Yersinia enterocolitica	ATCC 51871	Unknown
	ATCC 9610	Unknown
	CMCC 52204	Unknown
Yersinia pseudotuberculos	ATCC 4284	Unknown
Yersinia pestis	CMCC 52001	Unknown
Salmonella typhi	ATCC 14028	Unknown
Escherichia col	ATCC 25922	Unknown
Shigella dysenteriae	CMCC 51630	Unknown
Shigella flexneri	ATCC 12022	Unknown
Shigella sonnei	ATCC 29930	Unknown
Serratia marcescens	ATCC 8100	Unknown
Klebsiella oxytoca	ATCC 13182	Unknown
Klebsiella pneumoniae	ATCC 13883	Unknown
Hafnia alvei	ATCC 29927	Unknown
Citrobacter freundii	ATCC 8090	Unknown
Proteus mirabilis	ATCC 25933	Unknown
Proteus vulgaris	ATCC 13315	Unknown
Listeria monocytogenes	ATCC 15313	Unknown
Bacteroides fragilis	ATCC 25285	Unknown
Lactobacillus acidophilus	ATCC 4356	Unknown
Staphylococcus aureus	ATCC 43300	Unknown
*Enterococcus avium*0	ATCC 14025	Unknown
Enterococcus faecalis	ATCC 29212	Unknown
Pantoea agglomerans	ATCC 27155	Unknown

ATCC, American Type Culture Collection, Rockville, MD, USA.
CGMCC, China General Microbiological Culture Collection, Berjing, P. R. China.
CMCC, National Center For Medical Culture Collections, Beijing, P.R.China.
The others were isolated by CIQ Tianjin.

2.6. Sensitivity of CPA detection in the genomic DNA

The concentration of *E. sakazakii* genomic DNA was tested by BioSpec-mini (Shimadzu Corporation, Japan) according to the protocol of the instrument (After the self-test of the instrument, adjust the base line with the solvent according to the DNA sample at 260 nm. Then, remove the blank control, test the DNA solution under the same conditions. The concentration could be read by the instrument directly.). To study the sensitivity of CPA reaction on the DNA solution, ten-fold serial dilutions (10^0 ng–10^{-2} fg) of total genomic DNA extracted from *E. sakazakii* were subject to CPA and PCR in triplicate according to “Materials and Method” 2.2, 2.4 and