WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 9, 543-549. Research Article ISSN 2277– 7105

COMPUTATION AND *IN-SILICO* VALIDATION OF CANDIDATE PRIMERS FOR PCR-FREE ON-SITE DETECTION OF ENTEROTOXIGENIC *ESCHERICHIA COLI*

Rajesh Singh Tomar and Anurag Jyoti†

Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior-474 005, India.

Article Received on
07 March 2018,

Revised on 27 March 2018,
Accepted on 17 April 2018

DOI: 10.20959/wjpr20189-11704

***Corresponding Author**
Anurag Jyoti
Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior-474 005, India.

ABSTRACT

Over population, industrialization and urban crowding has led to the pollution of water resources resulting into the outbreaks of diarrheal diseases, threatening human lives. Enterotoxigenic *Escherichia coli* (ETEC) is a potential pathovar responsible for water-borne diarrhea among children and travelers. Rapid and on-site detection of ETEC is important for the management of these diseases. This creates a selection pressure on the scientists to develop a potential detection tool to diagnose the causative agent. The present study attempts to design and *in-silico* validate the specific candidate primers of isothermal amplification to detect ETEC. The primers for isothermal amplification were computed targeting *LT1* signature gene of ETEC. A web-based dedicated software Primer Explorer V4 was used to compute the primers. All the primers were further checked for the inclusivity and exclusivity in nucleotide-BLAST. Results showed that all the primers are highly specific towards their respective targets. Therefore, the present study implicates the computation of primers of PCR-free isothermal amplification for the detection of ETEC.

KEYWORDS: Enterotoxigenic *Escherichia coli*, Nanoarray, Isothermal amplification, point-of-care diagnostics.

INTRODUCTION

Presence of pathogenic microbes in potable water adversely impacts human health and sustainable development world wide. Rapid urbanization has converted environmental reservoirs as the dump site for wastes. Potable water resources are heavily contaminated with

the industrial effluents.[1] Such water resources harbor deadly pathogens. Enterotoxigenic *Escherichia coli* (ETEC) is one of the most prevalent pathogens in these resources.[2,3,4,5]

The detection of water-borne disease outbreaks help in identifying the origin of disease, the causative agents and the source involved. Conventional methods for detection of ETEC rely on the cultivation in appropriate culture media and further biochemical characterization. These methods are laborious, non-specific and time consuming.[6,7] The molecular approach, Polymerase Chain Reaction (PCR) has been validated by International Organization for Standardization (ISO) and is now used for testing of water-borne pathogens.[8] The sensitivity of the PCR is often not sufficient and post PCR processing is needed. Additionally, PCR requires technical equipment and laboratory setup, which is not suitable for on-site-diagnostics. Real Time PCR or quantitative PCR (q-PCR), more sensitive than conventional PCR, is being applied in the environmental risk assessment.[2] Although the method is sensitive, expensive instrumentation is required.[9]

Nanotechnology has been explored for the detection of nucleic acid of pathogens.[10] After hybridization with the target DNA, the gold nanoparticle functionalized oligonucleotides, exhibit a distinct color change (from red to purple) due to aggregate formation and red shift in plasmon band.[11] The assay is fast and simple, but still needs the PCR amplified products as target. To overcome the need for PCR with its complex temperature program, other DNA amplification techniques required. The Loop-mediated isothermal amplification (LAMP) is a promising gene amplification technique, in which the reaction can be processed at a constant temperature. The assay can be termed as “one-temperature DNA amplification" for a "one-pot detection" of a particular bacterium. In the LAMP method four primers are used to recognize six different locations on the target DNA, thus the specificity is extremely high.[11] By using this method, a few copies of the target DNA is specifically amplified to as high number as 10^9 within 1h under the isothermal (55-65^{0}C) conditions.[12] The amplicons are detected by measuring the visible turbidity caused by increasing quantity of Magnesium pyrophosphate in solution.[13,14] The sensitivity and specificity of this method has been demonstrated for detection of bacteria in samples.[15]

The present study aims to compute and *in-silico* validate the specific candidate primers of isothermal amplification to detect ETEC.

MATERIALS AND METHODS

Gene sequence retrieval from database

Enterotoxigenic *Escherichia coli*, pathovar responsible for the water-borne travelers' diarrhea was selected to compute primers for Loop-mediated isothermal amplification assay for detection in water samples. Nucleotide sequence of signature virulent gene *LT1* of ETEC was retrieved from the GenBank of NCBI, U.S.A.

Table 1: GenBank sequence identification number of *LT1* gene.

Microorganisms/ Genes	Accession number of gene sequence
Enterotoxigenic *Escherichia coli*	
LT1	AB011677

Primer designing for LAMP assay

The complete domain nucleotide sequence of *LT1* gene, unique to ETEC, was used to compute candidate oligonucleotide primers using Primer Explorer V4 software. Various parameters were considered for designing the primers. These include; Tm (melting temperature), % GC content, secondary structure, distance between primers etc.

Inclusivity and Exclusivity of designed primers

Computed primers were checked for their inclusivity and exclusivity against the sequenced microbial genomes in nucleotide-BLAST. This ensures the specificity of primers towards target pathogen.

In-Silico analysis of primers

In-silico PCR was performed to check the correct amplification of target DNA. Selected primer pairs were allowed to amplify and detect the *LT-1* gene of ETEC using this web-based software.

RESULTS

Computation of candidate oligonucleotides targeting Enterotoxigenic *Escherichia coli*

Highly specific primers for LAMPCR were computed using web based primer computational software, Primer Explorer V4. The individual primers varied from 21 bases to 25 bases long. The outer primer pairs F3-B3 generated a PCR product of 203 bp. Similarly primer pair F2-B2 generated amplicons of 155 bp. Other primers F1P and B1P were generated by combining F1c and F2 and B1c and B2 respectively (Table 2).

Table 2: Computed primers of the LAMP study for detection of ETEC.

label	5'pos	3'pos	length	Tm	5'dG	3'dG	Sequence
F3	982	1005	24	56.94	-6.34	-4.31	CGCAACACACAAATATATACGATA
B3	1164	1184	21	56.52	-5.53	-4.06	GTCTCGGTCAGATATGTGATT
FIP			50				GCCGCTCTTAAATGTAATGATAACC-TGACAAGATACTATCATATACGGAA
BIP			42				AGTCCCGGGCAGTCAACATA-TTAATGTGTCCTTCATCCTTTC
F2	1008	1032	25	56.73	-4.58	-5.69	TGACAAGATACTATCATATACGGAA
F1c	1056	1080	25	60.14	-7.76	-3.73	GCCGCTCTTAAATGTAATGATAACC
B2	1141	1162	22	55.74	-2.90	-4.46	TTAATGTGTCCTTCATCCTTTC
B1c	1098	1117	20	62.87	-5.69	-3.29	AGTCCCGGGCAGTCAACATA

Specificity of LAMPCR primers

Results showed that all the primers are highly specific towards their respective targets. E-value of the BLAST result was found to be very low for the target gene. There was no similarity found in other bacterial gene or genome.

***In-Silico* validation of designed candidate oligonucleotides**

In silico PCR amplification study revealed that the primer pairs amplify desired products of the target gene *LT-1* of ETEC.

DISCUSSION

Virulence genes are present in the pathogenic islands of a bacterial species. These are essential for the survival and pathogenicity of bacteria. These essential genes are more evolutionary conserved than non-essential genes in bacteria.[16] Present study aimed to compute the candidate oligonucleotides for the PCR-free detection of enterotoxigenic *Escherichia coli*. The pathogenicity of ETEC is directly related to the *LT1* gene expressing the enterotoxin LT.[18] Disease burdens due to these pathogenic bacteria are attributes to poor hygiene and poor management of water resources. This can be diminished by early and accurate diagnostic techniques. Rapid detection of these organisms within few hours desires development of sensitive and specific methods. Quantitative PCR has improved the detection system by use of specific probes and instrumentation. Although the method is highly sensitive, it still requires expensive instrumentation.[9]

PCR-free detection of pathogenic bacteria is one of the important and striking features of Loop-mediated isothermal amplification. Proper execution of the amplification needs careful computation of the primers and their *in-silico* validation. As a result, the time needed for *in-*

vitro optimizations will be minimized. Primers were computed to target template at different positions.

Inclusivity and exclusivity study revealed that the computed primers are highly specific towards their target. Expect value (E-value) and score from the BLAST output were analysed for each primer. E-value was found to be minimum and score was found to be maximum for all the primers. *In silico* PCR amplification study was performed to check the formation of desired products of *LT-1* gene of ETEC. The results revealed that the primer pairs amplify desired products of the target gene *LT-1* of ETEC.

CONCLUSION

In conclusion, the *in-silico* amplification of the target gene prior to bench optimization for detection of ETEC can minimize time and cost needed for *in vitro* verification of work. Such validated economically viable LAMPCR based assay could be used for the management of water quality as well as outbreaks.

ACKNOWLEDGMENT

We wish to express our sincere acknowledgement to Dr. Ashok Kumar Chauhan, President, RBEF parent organization of Amity University Madhya Pradesh (AUMP), Dr. Aseem Chauhan, Additional President, RBEF and chairman of AUMP; Lt. Gen. V.K. Sharma, AVSM (Retd.), Vice Chancellor of AUMP, Gwalior, for providing their valuable support, necessary facilities and encouragement throughout the work.

REFERENCES

1. Tomar RS, Jyoti A. A novel duplex PCR for detection of *Salmonellae* from drinking water samples. PHARMANEST - An Internat. Journ. Advan. Pharma. Sci., 2017; 8(3): 1-6.
2. Ram S, Vajpayee P, Shanker R. Rapid culture-independent quantitative detection of enterotoxigenic *Escherichia coli* in surface waters by real-time PCR with molecular beacon. Environ. Sci. Technol., 2008; 42: 4577.
3. Bavaro MF. *Escherichia coli* O157: what every internist and gastroenterologist should know. Curr. Gastroenterol., 2009; 11: 301.
4. Gonzales-Siles L, Sjöling Å. The different ecological niches of enterotoxigenic *Escherichia coli*. Environ. Microbiol., 2016; 18(3): 741–751.

5. Jyoti A, Singh SP, Yashpal M, Dwivedi PD, Shanker R. Rapid detection of enterotoxigenic *Escherichia coli* gene using bio-conjugated gold nano-particles. Journ. Biomed. Nanotechnol., 2011; 7: 170.
6. Deshmukh RA, Joshi K, Bhand S, Roy U. Recent developments in detection and enumeration of waterborne bacteria: a retrospective minireview. Microbiol. open., 2016; 5(6): 901–922.
7. Jyoti A, Tomar RS. Detection of pathogenic bacteria using nanobiosensors. Environ. Chemist. Lett., 2016; 15: 1-6.
8. Malorny B, Lo¨fstro¨m C, Wagner M, Kra¨mer N, Hoorfar J. Enumeration of salmonella bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. Appl. Environ. Microbiol., 2008; 74: 1299.
9. Javier DJ, Gonzalez AC, Weigum SE, White Jr. AC, Kortum RR. Oligonucleotide-gold nanoparticle networks for detection of *Cryptosporidium parvum* heat shock protein 70 mRNA. J. Clin. Microbiol., 2009; 47: 4060.
10. Costa P, Amaro A, Botelho A, Ina´cio J, Baptista PV. Gold nanoprobe assay for the identification of mycobacteria of the *Mycobacterium tuberculosis* complex. Europ. Soc. Clin. Microbiol. Infec. Dis., 2010; 16: 1464.
11. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. Nucl. Acids Res., 2010; 28: e63.
12. Lu Y, Yang W, Shi L, Li L, Alam MJ, Guo S, Miyoshi S. Specific detection of viable Salmonella cells by an ethidium monoazide-loop mediated isothermal amplification (EMA-LAMP) method. J. Heal. Sci., 2009; 55: 820.
13. Li Y, Fan P, Zhou S, Zhang L. Loop-mediated isothermal amplification (LAMP): A novel rapid detection platform for pathogens. Microb. Pathogen., 2017; 107: 54-61.
14. Wan L, Chen T, Gao J, Dong C, Wong AHH, Jia Y, Mak PI, Deng CX, Martins RP. A digital microfluidic system for loop-mediated isothermal amplification and sequence specific pathogen detection. Sci. Rep., 2017; 7: 1-11.
15. Kinoshita Y, Niwa H, Katayama Y. Use of loop-mediated isothermal amplification to detect six groups of pathogens causing secondary lower respiratory bacterial infections in horses. Microbiol. Immunol., 2015; 59: 365–370.
16. Niu C, Yu D, Wang Y, Ren H, Jin Y, Zhou W, Li B, Cheng Y, Yue J, Gao Z, Liang L. Common and pathogen-specific virulence factors are different in function and structure. Virulen., 2013; 15: 473-82.

17. Cajetan ICI, Ikeneche NF, Bassey BE, Al-Gallas N, Casmir AA, Nnennaya IR. Characterization of Toxins and Colonization Factors of Enterotoxigenic *Escherichia coli* Isolates from Children with Acute Diarrhea in Abuja, Nigeria. Jundish. J Microbiol. (In Press): e64269.